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OXYGEN ISOTOPE STUDIES IN SULPHATE

by



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Abstract

This thesis attempts for the first time to understand some aspects of oxygen and sulphur isotope fractionation during the reduction of sulphate to sulphide by synergetic pairs of organisms.

The reduction of sulphate by graphite was examined and careful attention to detail has significantly improved the reproducibility and precision of oxygen isotope measurements in sulphate. The author also contributed to the program of evaluating isotopic abundance ratios by the application of a PDP-8 computer to the ion current measurement system of the mass spectrometer.

Oxygen and sulphur isotopic abundance ratio determinations for unreacted sulphate and sulphur data for the H_2S product were made during four synergetic reductions of sulphate by *Bacillus* 8P and *Clostridium* Dm3 . Both normal and inverse kinetic isotope effects were observed. Data for the unreacted sulphate yielded a 3.82 ± 0.05 ratio for the $\delta S^{34} : \delta O^{18}$ values. No correlation was observed between the H_2S final product and the sulphate data because of a build up of intermediates and the fastidious nature of these organisms. Despite recent evidence that the δO^{18} value of the unreacted sulphate is related to the isotopic composition of the water, it is believed that this approximately 4:1 enrichment ratio has particular significance.

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CHAPTER I OXYGEN ISOTOPE ABUNDANCES IN SULPHATE

1.1 Introduction

Oxygen isotope studies are proving to be one of the most useful tools for studying the environment and the interactions of rocks, minerals, and water over a wide range of geological conditions. Most of the major oxygen-bearing mineral groups have been studied in detail, such as the carbonates, silicates, and oxides. One of the common rock forming mineral groups which has received little attention yet is present in many physical, chemical, biological, and geological processes, is the sulphate group.

Historically several analytical procedures have been developed for the extraction of oxygen from silicates and other oxygen compounds for precise isotopic analysis. Such studies often make it possible to define environments with greater detail than conventional techniques. In particular, the oxygen and sulphur isotope abundance ratios in sulphate reveal information concerning processes in the oxygen and sulphur cycles in the ocean-atmosphere system.

Since the work of SZABO, TUDGE, MACNAMARA and THODE (1950), variations in the S^{34}/S^{32} abundance ratios in sulphates have been routinely measured for over twenty years. It has long been realized that complementary O^{18}/O^{16} abun-

dance data could assist in the interpretation of sulphate geochemistry and biochemistry. However, it has only been during the past few years that investigators have carried out extensive oxygen isotope analyses.

1.2 Sulphate Ion Oxygen Isotope Exchange

Because sulphates are important primary and secondary minerals in sedimentary rocks, and in addition can be found in igneous and metamorphic rock systems, it became necessary to learn more of the fundamental oxygen isotope behavior. If sulphate is to be recovered from a variety of solutions and mineral assemblages, it is necessary to know what exchange takes place under experimental conditions involved in the recovery of sulphate for oxygen isotopic measurements.

The early work of TEIS (1956) indicated that oxygen isotopes exchanged very slowly between dissolved sulphate and water in near neutral solutions at earth surface temperatures. For example, the half period for exchange of Na_2SO_4 in neutral solutions was found to be about 70 years, the full period for near equilibrium being reached in about 450 years. HOERING and KENNEDY (1957) pointed out, however, that the exchange between sulphate and water was accelerated considerably in concentrated acid solutions. Under quite adverse chemical conditions, RAFTER (1967) found negligible

alteration to the isotopic composition of sulphate during extraction procedures which would be encountered during routine laboratory analyses.

Confirming the general observations of TEIS (1956), LLOYD (1968) found the exchange rates of sulphate and water were very slow in normal geological environments (97 per cent exchange of oceanic sulphate with ocean water having a mean temperature of 4°C and a pH of 8.2 would require of the order of 250,000 years).

TEIS (1956) also examined the exchange of sodium sulphate and carbon dioxide gas. At 900°C , the half period was found to be 2.2 hours and by extrapolation to ordinary temperatures (about 20°C), the half period of exchange was estimated to be slightly more than 10,000 years.

Thus it can be concluded that in most experimental and naturally occurring procedures involving the sulphate ion, the oxygen isotopic exchange rates are extremely slow in sulphate - water and sulphate - CO_2 systems. At earth surface conditions, it may require about 10^3 to 10^5 years to approximate near oxygen isotope exchange equilibrium. With such a slow exchange, one would expect the isotopic values of the sulphate to reflect a long term average environmental condition rather than the "instantaneous" environmental conditions. As such, sulphate can be extracted in the laboratory without fractionation of the oxygen isotopes.

1.3 Terrestrial Variations of Oxygen Isotope Abundances in Sulphates

The oxygen isotopic abundance variations of sulphate in nature are dependent upon the history of the sulphate ion exchange in the sulphur cycle. HOLSER and KAPLAN (1966) calculated the time of residence for sulphur in the sea to be about 21 million years. This would be equivalent to more than 80 half-times of exchange. Therefore one would probably expect oceanic sulphate to be in isotopic equilibrium with ocean water. But if the oxidation-reduction turnover of sulphate is responsible for preventing the establishment of isotopic equilibrium, then from HOLSER and KAPLAN (1966), about 30 per cent of the sulphate oxygen passes through the sulphur cycle over a time period probably less than 50,000 years. This represents more than one half of the oxygen found in the present atmosphere, and hence suggests that the sulphur cycle could be one of the important factors regulating the oxygen balance in the ocean-atmosphere system.

With the development of a quantitative and reliable method [RAFTER (1967), LONGINELLI and CRAIG (1967), and LLOYD (1967)] for the extraction of oxygen atoms from sulphate for precise isotopic analysis, the complementary O^{18}/O^{16} isotopic ratios opened up unlimited and almost totally unexplored possibilities for the study of oxygen isotope abundance

ratios for sulphate in geochemical and biochemical environments. The purpose of the research turned towards being able to know the range of isotopic composition of the dissolved sulphate, to check the possible existence of some relation between the oxygen isotopic composition of sulphate and that of the waters in which it is found, and to obtain information of the origin of the sulphate and its history.

RAFTER and MIZUTANI (1967a) examined the oxygen isotopic abundance ratios of sulphate and water from Lake Vanda in the Antarctic. The S^{34}/S^{32} ratio in the sulphate was found to increase approximately four times faster than the O^{18}/O^{16} ratio in the sulphate with depth. Also the O^{18}/O^{16} ratio in the sulphate increased at twice the rate as in the water. A problem arose concerning interpretation. The assumption made at that time was that biological fractionation caused such an enrichment. The establishment of such a correlation for sulphur-34 and the oxygen-18 content of these sulphates led to more extensive work in an attempt to understand the variations of oxygen-18 in naturally occurring sulphates.

LONGINELLI (1968) found no relation between the O^{18}/O^{16} ratios of sulphate and water and temperatures in waters from 34 thermal springs in Tuscany (Italy). CORTECCI and LONGINELLI (1968) found a positive correlation existed for oxygen-18 in sulphate and some lake waters. The results

obtained were concluded to be probably caused by several different factors - one of these factors being a wide spread microbiological activity. Later, LONGINELLI and CORTECCI (1969) found, for two rivers, variations almost always in a direction of positive enrichment in the heavy isotopes (the oxygen-18 and sulphur-34). In contrast to RAFTER and MIZUTANI (1967a), a comparison of S^{34}/S^{32} to O^{18}/O^{16} yielded a slope which varied from 0.6 to 0.7 with time.

LLOYD (1968) did attempt some bacterial reductions of sulphate, but encountered difficulties. It was found difficult to control the rate at which the bacterial reduction progressed. Also the medium would become poisoned with excess hydrogen sulphide after a time, and the reduction would stop. Thus none of the experiments went to completion. From the data obtained with both pure cultures and natural mixtures of bacteria, LLOYD (1968) did indicate that there was a preferential consumption of oxygen-16 by the bacteria.

MIZUTANI and RAFTER (1969) attempted to establish the relationship between sulphur-34 and oxygen-18 values in the sulphate employed for the study of the bacterial reduction of sulphate. They also wished to test the relationship between the oxygen-18 values of sulphate and the water in which the sulphate was formed during the bacterial oxida-

tion of sulphur. In the bacterial reduction of sulphate, MIZUTANI and RAFTER (1969) found that at any stage of the reduction, the remaining sulphate was enriched in both oxygen-18 and sulphur-34. The ratio of the sulphur-34 enrichment to the oxygen-18 enrichment was approximately 4:1. In the bacterial oxidation of sulphur, very little difference was observed between the oxygen-18 value of any remaining sulphate and that of the water in which the sulphate was found. Such a correlation was most probably the result of the hydrogen sulphide formed from the bacterial reduction becoming available for the bacterial oxidation to sulphate.

The oxygen and sulphur isotopic abundance ratios of some gypsums and evaporites were reported by SAKAI (1972). This work discussed the relationship between the two isotopic species in terms of geological ages of samples from Precambrian to the present. A somewhat similar study was performed by LLOYD (1973), where he analyzed interstitial water from cores. The latter study only presented data for the oxygen in the sulphate, but no meaningful conclusions were forthcoming. The isotopic composition with depth was very interesting, but further work is necessary before meaningful conclusions can be made.

1.4 Present Investigation

At the outset of this project, investigations of O^{18}/O^{16} abundance ratio in sulphates left the following not completely understood:

- (a) the terrestrial range of oxygen isotope variations in sulphates,
- (b) the extent and rate of exchange of oxygen atoms,
- (c) the kinetic isotope effects involved in the production and reduction (chemical and biological) of sulphates, and
- (d) the lack of reproducibility in oxygen isotopic abundance data.

In many cases not only isotope effects, but also basic mechanisms involved were not fully understood. For example, the complex processes of sulphate reduction by bacteria are not yet fully comprehended.

In the present investigation, it was decided to pursue two problems. The first was an examination of the reduction of sulphate by graphite with the view of obtaining consistent oxygen isotope abundance data (Chapter II). The second problem was to effect oxygen isotope fractionation during bacterial sulphate reduction and to compare any effects observed to those obtained during the sulphur isotope fractionation (Chapter IV). The resultant effects

found in both cases would be used as an aid in the understanding of the mechanisms involved in the microbiological reduction of sulphate (Chapter V).

As seen in Section 1.3 , there were independent developments during the course of the present investigation which are related to the problem selected. However, the approaches taken and the emphasis of the present work are markedly different. For example, MIZUTANI and RAFTER (1969) observed isotope effects during microbiological sulphate reduction under experimental conditions quite different from those of the present work. They utilized several flasks of natural mud and sea water. A given flask was opened at a specified time and its contents examined isotopically. The experiments of the present investigation contrasted to those conditions in several ways. Pure isolates of a specific organism were used as opposed to the complex natural mixture of organisms employed by MIZUTANI and RAFTER (1969). The medium used for the present study was very simple in contrast to the unknown complex medium constituting their "mud".

In any one of the experiments undertaken, one large reaction vessel was used (rather than many flasks), gaseous products were constantly flushed and collected, and the liquid contents periodically examined. Thus, the present investigation was able realistically to evaluate chemical and isotopic balances. The experimental conditions

of MIZUTANI and RAFTER (1969) were quite relevant to the terrestrial situation. On the other hand, the present approach was more orientated towards elucidating fundamental microbiological mechanisms involved in the bacterial reduction of sulphate.

CHAPTER II GRAPHITE REDUCTION OF SULPHATE TO CARBON DIOXIDE

2.1 Review

It is relatively simple to extract oxygen atoms from the sulphate ion and react them with graphite. However, it is very difficult to effect 100 per cent conversion to carbon dioxide, and to avoid oxygen isotope exchange with other molecules. Both of these difficulties must be overcome in order to determine the oxygen isotopic composition of sulphates with good reproduceability.

It would appear that the techniques employed for the oxygen extraction from sulphate have paralleled those used for determining the oxygen isotope abundances in silicates and other oxygen bearing compounds.

One of the pioneering efforts in this regard was conducted by MANION, UREY and BLEAKNEY (1934). In their determination of oxygen isotope abundances in silicates, a mixture of the silicates and carbon was reacted with carbon tetrachloride at 1000°C , whereupon the carbon monoxide formed was converted into water by combustion with hydrogen. The oxygen obtained by the electrolysis of the water was then analyzed. Somewhat later, ATEN and HEVESY (1938) reduced sulphate at 900°C with some finely ground, sub-ignited coal. In this determination, the mixture of CO_2 -CO- H_2 which formed

was converted into water, over a nickel catalyst at 350°C. The resultant electrolysis then yielded oxygen which was analyzed.

From that time until recently, the analytical procedures for the extraction of oxygen were all based on one of the following two types of reactions;

- (1) the reduction by carbon at high temperatures (1600 to 2000°C) to yield carbon monoxide, and
- (2) the oxidation by fluorine or some halogen fluoride to yield molecular oxygen.

The carbon reduction technique was further modified by HALPERIN and TAUBE (1952) in their studies of oxygen isotope abundances in barium sulphate. They used five times as much graphite as sulphate, where this mixture was placed in a platinum crucible and raised to 1000°C by induction heating. About 75 per cent of the oxygen was converted to CO₂ and the remainder to CO. However, only the CO₂ was analyzed. The reduction method was refined by SWANDER (1953) who converted the oxygen of silicate minerals and rocks to CO in a thermal reaction



which was quantitative when powdered silica was mixed with graphite and heated in a vacuum to 2000°C. However, when

mixed samples containing alkali metals, alkali earths or aluminum were employed, oxygen yields were accompanied by large isotope fractionations. This method was modified again by CLAYTON and EPSTEIN (1958) to determine the isotopic composition of oxygen in natural oxides of silicon and iron. The carbon monoxide formed was converted to CO_2 using a nickel catalyst and then analyzed in the mass spectrometer. Spectrographically pure graphite was used by VINOGRADOV, DONTSOVA, and CHUPAKHIN (1958) as well as DONTSOVA (1959), to decrease the effects of fractionation previously found. In particular, this improvement now made available a reliable analysis of the alumino-silicates, which had not yet been successfully analyzed by the graphite method of reduction.

A few isotopic analyses of some naturally occurring sulphates were carried out by TEIS (1956) who reduced the sulphate at 900°C with some finely ground coal, and converted the $\text{CO}_2 + \text{CO} + \text{H}_2$ so formed into water over a nickel catalyst at 350°C , which was then analyzed for oxygen-18. It appears that no further work on the carbon reduction method for the recovery of oxygen as the more stable carbon dioxide was published until 1967.

On the other hand, oxidation techniques were employed by SILVERMAN (1951), and BAERTSCHI and SILVERMAN (1951), to yield molecular oxygen quantitatively from sili-

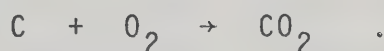
cate rocks. Two methods developed by these studies were:

(1) the employment of chlorine trifluoride and hydrogen fluoride at 430°C , and

(2) fluorine and hydrogen fluoride at 420°C .

For most rocks and minerals, these were sufficient but for basic and ultra-basic rocks, the yield was about 80 per cent.

TUDGE (1960) later used chlorine pentafluoride for orthophosphate and condensed phosphates. The product, molecular oxygen, was more manageable when converted to CO_2 , by the reaction



CLAYTON and MAYEDA (1963) as well as LONGINELLI (1965) used bromine pentafluoride for silicates and phosphates, respectively, since it was easier and safer to handle than fluorine. Also it reacts with some minerals which do not react completely with fluorine.

Therefore, by the mid-sixties, the carbon reduction and the fluoride oxidation processes were well established for the quantitative extraction of oxygen from silicates and other oxygen compounds for the determination of $\text{O}^{18}/\text{O}^{16}$ ratios. These techniques evolved with a preference for the product CO_2 and not CO . This was more suitable, since;

- (1) CO is not stable and will disproportionate into CO_2 plus C, whereas CO_2 is stable and only reduces in part to CO in the presence of carbon at a high temperature (above 900°C),
- (2) CO_2 is less of a health hazard than carbon monoxide,
- (3) CO and N_2 have the same molecular mass range (28) and cannot be separated in the mass spectrometers usually employed in isotope abundance studies, and
- (4) CO requires higher temperatures to be the major product of a reduction ($1600\text{--}2000^\circ\text{C}$) whereas CO_2 becomes the major product at lower temperatures (about 1000°C).

LLLOYD (1967) ground sulphate samples with spectrographically pure graphite in excess (ten-fold). Then a pellet of the mixture was formed. This was heated in a graphite crucible to 1000°C by an induction heater with both carbon dioxide and carbon monoxide being the gaseous product. The gas so formed was passed through a nickel catalyst furnace where the carbon monoxide was converted to carbon dioxide, and the total CO_2 product then collected by freezing in liquid nitrogen. LLLOYD (1967) also found that the most important factor in obtaining good yields and isotopic reproducibility,

was that the sulphate should be in the form of barium sulphate. This is because other sulphates (such as calcium sulphate) will produce SO_2 and COS in addition to CO and CO_2 .

AGGETT, BUNTON, LEWIS, LLEWELLYN, O'CONNOR, CHARMAIN, and ODELL (1965) made use of the catalytic conversion of carbon monoxide to carbon dioxide through utilization of the equilibrium



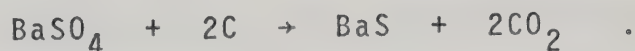
for isotopic analysis of oxygen in organic compounds. They also pointed out that at low pressures and low temperatures this reaction would normally be too slow, since formation of CO_2 is favored by low temperature and high pressure. This difficulty can be best overcome by the use of a high voltage discharge between two parallel plates. These are most effective, when used in a vessel partially immersed in liquid nitrogen so that the CO_2 can be condensed as soon as it is formed.

LONGINELLI and CRAIG (1967) precipitated sulphate as barium sulphate and dried it for 3 hours at 120°C in a vacuum furnace. The barium sulphate was reduced with excess spectrographic graphite (ten to fifteen-fold) by induction heating to 1100°C in a graphite crucible with lid. Previous to this, however, the sample had been degassed at a few hundred degrees for fifteen minutes before being reduced at

elevated temperature for 45 minutes. Any CO_2 that was formed was continually condensed, since the heating chamber was connected to a liquid nitrogen trap. The residual CO was converted to CO_2 by sparking with a Tesla coil above a cold trap, using two platinum sheets as electrodes.

RAFTER (1967) also used barium sulphate, however intimately mixed with the pure graphite. This mixture was placed in platinum boats with lids, and initially outgassed at 500°C *in vacuo*, then heated to 1100°C in a micro-furnace. As the two products formed, the CO_2 condensed in the first nitrogen cold trap but the CO formed was passed on to a discharge tube with copper plates, immersed in a cold trap to condense the CO_2 formed during the discharge reaction. The two condensed samples were then combined for mass spectrometric analysis.

RAFTER (1967) felt that a lower temperature range (about $900\text{--}1050^\circ\text{C}$) was adequate for the graphite reduction of sulphates of sodium, potassium, calcium, strontium, and barium. This work realized a higher production of carbon dioxide at these lower temperatures, the reaction being essentially



This investigation showed that at elevated temperatures the carbon dioxide-graphite reduction was indeed measurable. After

three hours at 1000°C , 12 per cent of the CO_2 had been reduced to CO. For sulphur isotope studies the recovery of sulphur as BaS is unaltered if there is any carbon monoxide present, but it is vital that all of the sulphate oxygen be recovered as carbon dioxide for oxygen isotope abundance measurements.

RAFTER and MIZUTANI (1967a) further modified the technique of RAFTER (1967) with respect to recovery refinements. They suspected that when the barium sulphate was heated over a gas burner in drying, some of the variability noted (± 0.29 ‰) was due to the reduction of this sulphate in the gas flame and its reoxidation by the atmospheric oxygen, or possibly an exchange reaction between the carbon dioxide in the gas flame and the sulphate. As a result, drying was thereafter a slow process over an electric hot plate, and only before weighing out portions for analysis. This change in technique led to somewhat more reproducible (± 0.18 ‰) results. It was this same reduction and subsequent reoxidation by atmospheric oxygen that prompted RAFTER (1967) to avoid filtration of the sulphate on filter paper before its ignition.

If the references are examined in detail, it is found that parameters other than temperature and pressure affect the ratio of the CO-CO₂ production. Whereas RAFTER (1967) obtained yields of 16 per cent CO and 75 per cent CO₂ with platinum crucibles, LONGINELLI and CRAIG (1967) found variable

yields from 98 per cent CO_2 with a new crucible, down to about 80 per cent after ten reactions using graphite crucibles. This latter technique reduced the reaction time for the



process. LONGINELLI and CRAIG (1967) verified this by heating carbon dioxide of known composition over the graphite crucible until about one half of it was reduced to carbon monoxide. The CO was sparked back to CO_2 and within the precision of the volume readings (to one per cent), all the CO_2 was recovered and the isotopic composition of the oxygen was unchanged (± 0.01 ‰). Gas chromatographic analysis further showed the final product of the sulphate reduction to be pure carbon dioxide when pure barium sulphate was used. (However, natural barite crystals resulted in the presence of a small amount of sulphur dioxide in the CO_2 .)

LONGINELLI (1968) noted a very significant factor, in that standard samples for intercalibration of the measurements are badly needed among the different laboratories. This is basically due to the experimental fact that different techniques of conversion to CO_2 of the CO produced in the reaction between barium sulphate and graphite can cause slight isotopic differences in the final samples.

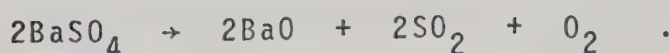
While the development of oxygen isotope studies in sulphate continued, SAKAI and KROUSE (1971) discussed the reduction technique further. This work pointed out significant memory effects, which were caused by oxygen isotope exchange between the product CO and the hot quartz walls. This effect is negligible when samples possessing a narrow isotopic spread are analyzed (for standard deviations of the order of ± 0.1 ‰). However, with sulphate of widely varying oxygen isotope compositions, the reproducibility of the isotopic determinations can deteriorate to greater than ± 0.5 ‰, despite satisfactory yields. Thus a systematic investigation of the sample preparation was necessary. SAKAI and KROUSE (1971) realized that this was not a problem of mass spectrometry, but rather a lack of precision in duplicating the oxygen isotope abundance ratio of the same sulphate sample. Their long term observations revealed that a tube of quartz, which displayed signs of devitrification after prolonged usage, produced larger memory phenomena than a relatively new tube. NORTHRUP and CLAYTON (1965) had earlier observed an oxygen isotope exchange between carbon dioxide and glass at temperatures above 250°C . This CO_2 , SAKAI and KROUSE (1971) noted, should not be an effective participant in exchange phenomena during the conversion. This is apparent, since it is rapidly frozen out of the system by the liquid nitrogen trap. But the other gaseous pro-

duct, carbon monoxide, does remain as a gas phase, and until converted would have a better opportunity to exchange oxygen isotopes with the hot quartz walls. Furthermore, one should not be misled by the amount of carbon monoxide which remains at the end of the heating cycle, since this represents only a fraction of the CO which actually forms during the earlier stages of the reaction. One possible solution involves a minimum of three subsequent conversions to assure the desired reproducibility for a particular sulphate sample (as was done in this thesis). Although the duplication of analyses should always be carried out, often in cases where the material is rare, only one measurement is possible. Thus the solution is really to eliminate memory effects. This is only a possibility if a more efficient CO conversion unit is developed. The simplest and most direct form of internal heating is that of making the platinum boat the location of highest resistance in an electrical circuit. This was done by SAKAI and KROUSE (1971) in conjunction with a water jacket around the quartz tube to greatly reduce oxygen isotope exchange with the quartz wall. This new apparatus showed no signs of memory effects when samples differing in oxygen-18 values by over 22 per mil were analyzed.

The complete procedure for the evaluation of the oxygen and sulphur isotope abundance ratios in the sulphates involves four possible operations. They are:

- a. the chemical precipitation of the sulphate in the preferred form of barium sulphate,
- b. the reduction of the BaSO_4 preferably to Ag_2S as the final product,
- c. the subsequent burning of the Ag_2S to form SO_2 , for mass spectrometric analysis of the sulphur isotope variations, and
- d. the reduction of the BaSO_4 to CO_2 for the oxygen isotope measurements.

All four of these procedures are reliable in that they cause no appreciable fractionation of the isotopes. The chief disadvantage, however, is the requisite time and labor. Such preparative procedures do constitute a major part of a research program which is focused on the study of sulphur and oxygen isotopes variations in sulphate. HOLT and ENGELKEMEIN (1970) described a method by which BaSO_4 is rapidly converted to SO_2 in one operation. Thus the time and effort which is consumed in the earlier techniques now can be drastically reduced. Here the barium sulphate is converted to sulphur dioxide by simply covering the sulphate with pulverized quartz powder in a fused quartz tube. Then in a vacuum it is heated to the softening point of quartz (1400°C), resulting in



The SO_2 produced is collected in a cold trap. The oxygen is pumped away and the BaO fuses with the silica surroundings. This method of conversion does not assure a uniform oxygen-18 abundance in the sulphur dioxide, as do previously discussed techniques, but a correction is used for the oxygen-18 interference in the mass spectrometric analysis. HOLT and ENGELKEMEIN (1970) observed no inherent source of interfering impurity associated with this thermal decomposition of pure BaSO_4 in the quartz environment.

Although the thermal decomposition of BaSO_4 did appear to reduce the sample preparation time and labor, the building of such an apparatus was not justified in view of the non-uniform oxygen-18 abundances encountered with this method. As a result, the proven laborious techniques were continued and the high voltage discharge conversion of CO to CO_2 retained.

2.2 Apparatus Used in the Present Study

Literature describing sulphate reduction processes continually avoids details of the operating procedures. Such methods were impossible to duplicate because of the lack of precision involved in defining seemingly unimportant but controversial steps when one attempts to follow such analyses. As a result, the following description of sulphate to CO_2 conversion will strive to clarify every detail of the method.

The high voltage discharge conversion unit, adopted for the conversion of CO to CO₂, was built into a vacuum system as shown in Figure 2-1. The reduction furnace (F) contained an element (E), made of self-bonded silicon carbide. The "Crusilite" (Norton Company, Worchester, Mass.) heating element was tubular in shape and had a 47 mm I.D. and 55 mm O.D. In the center of the 14" overall length, was a 6" "hot zone" (H). This region of high temperature was formed by cutting a spiral in the element such that the "cold ends" are not spiralled. This action produced a much lower resistance zone at either end of the element, with the center region of highest resistance. There were no mechanical joints so as to ensure no possibility of failure due to mechanical dissimilarities. The "Crusilite" elements were glazed to provide an increased resistance to oxidation. Because of this glaze, excellent temperature control and reproducibility was available - the element has not been replaced after four years of continual use. The glaze prevents rapid aging (at 1100°C the rate of resistance change was about 10 per cent per thousand hours, or an estimated 25 per cent per thousand hours at 1400°C).

Temperatures in excess of 1250° were realized when 20 amp at 100 v was applied. The power supply (20 amp, 140 v variac) used was directly wired to the element support sleeves at one end of the doubly spiralled muffle. This type was

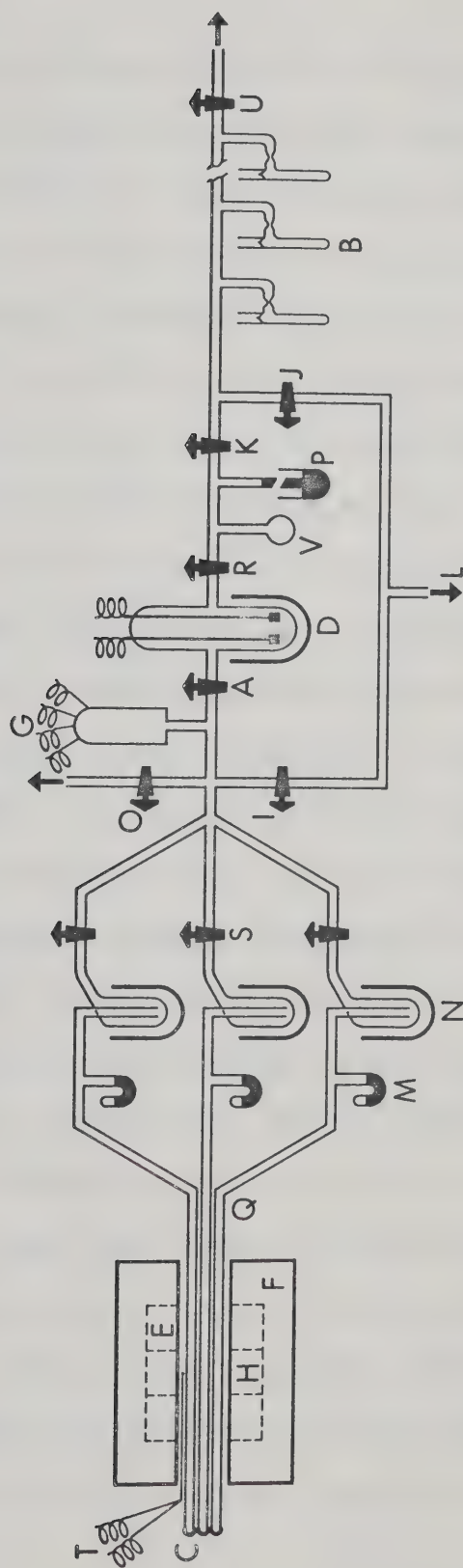


Figure 2-1 Apparatus for the graphite reduction of sulphate and the CO conversion to CO₂.

advantageous in that both electrical connections were at the same end, leaving free access to the other end. The element was mounted horizontally, thus allowing the freedom to expand and contract in the firebrick furnace. The element's terminal connectors protruded beyond the furnace face so as to permit good ventilation and prevent overheating.

A Pt-Pt (13 per cent Rd) thermocouple (T) was inserted in the "hot zone" (H) between the inner surface of the element (E) and the quartz tubes (Q) inside. Temperatures higher than 1250⁰C were only attempted once, since the quartz combustion tubes (Q) began to soften at these and more elevated temperatures. The three quartz tubes (Q) had ground glass caps (C) through which the specimen could be introduced to and removed from the "hot zone" (H) of the furnace. Temperature control was solely dependent upon the readings from the thermocouple temperature recorder (T).

The quartz tubes (Q) were each monitored qualitatively by a mercury manometer (M) attached to each combustion tube. A liquid nitrogen cold trap (N) was also connected to each chamber so that the carbon dioxide which formed was condensed as soon as it was formed in the respective quartz combustion chambers (Q). With stopcocks (S) closed, each sample in the system could be monitored by the respective pressure manometers (M). One of the three stopcocks (S) was usually

allowed to remain open, thus permitting the CO gas formed in that tube to expand further into the vacuum gauge head (G). As the reaction in the tubes proceeded, the manifold pressure reading could be obtained for one of the three samples being reduced. But this limitation was minimized by using specimens of about the same size. These manifold readings were most helpful in that they indicated the rate of CO production as well as when the formation had reached completion - no further decrease in the pressure as recorded by the vacuum gauge (G).

As the CO₂ formed, it was frozen down in the liquid nitrogen traps (N) immediately. The CO produced during the reduction still remained between the ground glass caps (C) and the stopcocks (S) - except for the monitored sample. This CO gas was now available for the conversion to carbon dioxide in the high voltage discharge conversion unit (D). With the vacuum gauge (G) turned off, stopcock A was then opened to allow the CO to enter the conversion unit (D). When the reaction



was completed, the vacuum head (G) was turned on to record a quantitative indication of the completeness of the conversion

and also the reduction of the sulphate specimen.

Once the CO to CO₂ conversion was finished, liquid nitrogen was placed around the calibration volume (V). When stopcock R was then opened, the CO₂ from the reduction could be transferred to this calibration volume. If stopcock R was subsequently closed and the cold liquid nitrogen bath removed, a volumetric calculation of the CO₂ gas can be made knowing the recorded manometer pressure (P), and the volume contained between the stopcocks (R and K) including the total volume when these stopcocks were closed. After the CO₂ volume was recorded, the stopcock K was opened allowing the gaseous CO₂ to expand towards the breakseal line (B). With the use of a liquid nitrogen bath, the sample was subsequently removed from the system, and was available for later mass spectrometric analysis. Both stopcocks I and J led to conventional pumping equipment (in this case, a mercury diffusion pump connected to a rotary vacuum pump). These were installed to permit the rapid evacuation of the apparatus.

High voltage for the CO to CO₂ conversion unit was supplied by a 4.5 Kv transformer. The electrodes of the unit were made of platinum, 2 cm by 4 cm, and 0.003 inch in thickness. The ground glass cap, through which the electrode leads passed, was removed when it was considered desirable to clean the platinum electrodes of accumulated carbon. It was found

necessary to cease sparking when a pressure reading was required to monitor progress of the reaction. Otherwise, the gaseous ionization excited the current carrying conductors of the pressure gauge, resulting in erroneous pressures.

Breakseals (B) were attached to the calibration volume (V) for the removal of the carbon dioxide produced by the graphite reduction of sulphate. To replace them, a stopcock (U) was opened to allow the breakseal line pressure to rise to atmospheric pressure. Stopcock (O) permitted the rapid replacement of samples to be reduced in the quartz combustion tubes. Both operations can be completed and the line evacuated with a minimum of disturbance to the rest of the system.

2.3 Method

About 50 mg of BaSO_4 (dried overnight at 120°C to expel any water present) was weighed accurately and then transferred to a small agate mortar. Following the same care in weighing and the transferral, about 50 mg of spectrographically pure graphite was weighed (after drying overnight at 900°C *in vacuo*) and transferred to the agate mortar. The specimen and graphite were then intimately mixed and transferred into a previously dried (overnight at 120°C) and weighed platinum boat.

This platinum boat was approximately 4 cm in length, 1 cm in depth, and about 1.5 cm wide. Lids, also platinum, were made for the boats and dried and weighed in the same manner as the boats.

The boat, containing the sample and graphite, was reweighed to obtain the exact weight of the mixture, since about 2 mg were lost during the mixing and transferral processes. The lid was then placed securely on the boat and its contents before another weighing of the sample. The lid always covered completely the boat and its contents, after a suggestion from LONGINELLI (private communication). This procedure gave consistently better yields.

The loss due to the mechanical mixing and transfer was usually of the order of 2 mg, independent of the sample size. This loss was most consistent throughout the entire work of this thesis. That is, as can be seen from Table 2-1, it was entirely the care given to the process that determined the amount of sample and graphite lost.

The boat, plus lid and contents, was then placed in one of the quartz combustion tubes (within the "hot zone") of the reduction furnace. That quartz tube was subsequently evacuated. This was done by first greasing the ground glass cap and then placing it over the quartz tube end after the

Table 2-1 Loss of graphite and sulphate due to
mixing and transfer

<u>Date</u>	<u>Boat</u>	<u>Weight (mg)</u>	<u>Before Mixing (mg)</u>	<u>After Mixing (mg)</u>	<u>Loss (mg)</u>
7/5/70	A	1393	1531	1529	2
	B	1278	1414	1413	3
	C	1428	1562	1562	0
13/5/70	A	1392	1520	1519	1
	B	1278	1410	1409	1
	C	1429	1569	1566	3
15/5/70	A	1392	1530	1531	0
	B	1278	1436	1436	0
	C	1429	1535	1534	1
22/5/70	A	1394	1534	1532	2
	B	1278	1431	1429	2
	C	1430	1539	1539	0

average loss = 1.3 mg

boat had been inserted. Now the appropriate stopcock (S) was opened after closure of stopcocks I and J, to ensure that the reduction chamber was most efficiently evacuated as well as maintaining the remainder of the vacuum system at about 0.001 Torr. Once the first specimen was introduced, the temperature was raised to 500°C from the stand-by temperature of 400°C . Thus any water vapor or gases produced or present in the tube were pumped away. Similarly two more specimens were prepared and introduced into the furnace and the evacuated system. The three samples were then left for about 15 minutes to ensure no residual gases were present. It was considered advisable at this stage of the reduction process to close stopcocks I and J to permit observation of the vacuum gauge reading (G) to detect the presence of any residual gas production.

Once no residual gas pressure was detected, dewar flasks containing liquid nitrogen were placed around the three CO_2 traps (N). If a combustion tube was to be monitored, as was always done, then a flask of liquid nitrogen must also be placed around the conversion unit (D). With two of the stopcocks (S) closed and the other open to the tube being monitored, stopcock A was checked to be open and R was now closed. Thus the monitor chamber was open to the vacuum gauge as well as the conversion unit. The furnace was then raised to 1100°C , which required approximately 25 minutes,

and was held at that temperature a further 15 minutes to ensure completeness of the reduction. It was turned down to 400°C once the reaction was finished.

As the carbon dioxide formed, it condensed in the liquid nitrogen traps (N). Any carbon monoxide produced was registered on the manometers (M). Always one of the three was the monitor - usually the first introduced into the quartz tubes. Thus the progress of the reduction (i.e. CO production) in that reaction tube was observed as the pressure in the quartz combustion chambers rose. When the pressure reached 0.05 Torr, the vacuum gauge was turned off to prevent erroneous readings (as well as possible damage to the vacuum tube circuit) when the high voltage discharge (D) was turned on. As evidenced by the visible gas glow, sparking was continued until it was ascertained that the CO to CO₂ conversion had proceeded to completion.

The two remaining samples were still contained by their stopcocks (S), since only one specimen could be converted to carbon dioxide in the discharge chamber at a time. The monitored pressure usually remained below 0.5 Torr throughout the experiment. The pressure was seen to drop markedly as the high voltage discharge enabled the

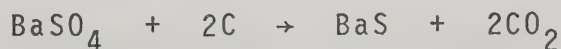


reaction to proceed. Here the discharge was terminated before the vacuum gauge (G) was turned on again, and vice versa, throughout the conversion of gaseous carbon monoxide to the stable carbon dioxide. It required about 15 minutes at elevated temperature, with alternate reading of the pressure, and sparking, until the residual gas was a sufficiently small fraction of the total CO_2 produced. This residual gas was then pumped away.

The liquid nitrogen dewar from the monitor CO_2 trap (N) was removed to allow the CO_2 condensed in the monitored trap (N) to evolve and condense in the discharge chamber, which at this time was still enclosed in a liquid nitrogen bath. Then the vacuum gauge (G) was turned off and the CO to CO_2 conversion unit discharge was engaged once more. This sparking time was extended considerably if the pressure in the system had risen even slightly after the transfer, thereby converting any final traces of carbon monoxide to carbon dioxide. This was easily noted by recording the vacuum head pressure before and after the evolution and subsequent discharge. Now that the reduction to gaseous carbon dioxide was complete, the conversion system (i.e. the monitor combustion tube, the vacuum gauge, and the CO - CO_2 conversion unit) was evacuated to less than 0.001 Torr. This CO_2 produced from the graphite reduction of

sulphate was hence ready for volumetric calibration and its subsequent extraction from the reduction line.

It should be noted that after three hours at 1000°C , about 12 per cent of the CO_2 produced would have been reduced to CO. Such a reduction would have occurred at the hot crucible containing unreacted excess graphite. This was avoided since the CO_2 traps (N), partially immersed in liquid nitrogen, forced the CO_2 to condense as soon as it was formed. (For sulphur isotope studies, the recovery of sulphur as BaS was unaltered if there was any CO present. But it was vital that all of the sulphate oxygen be recovered as CO_2 , if the equation



was to be used for the study of oxygen isotopes in sulphate.)

With the conversion system at 0.001 Torr and the CO_2 frozen in liquid nitrogen trap about the discharge chamber, another dewar of liquid nitrogen was placed around the calibration volume (V). Then the CO_2 gas, produced from the graphite reduction of sulphate, was allowed to expand into the calibration volume after closing stopcock K and opening stopcock R. On removal of the cold trap around the conversion unit, the gas evolved into the calibration volume where it was immediately condensed. After the transferral was complete, stopcock R was closed and this cold trap also removed so that the CO_2 could

then expand, thus allowing a calibration of the volume of gas evolved. This manometer reading gave a preliminary check on the completeness of the reduction. A liquid nitrogen flask was placed under a breakseal before the gas was transferred to the breakseal line by the opening of stopcock K. Once the product of the reduction was frozen down in the breakseal (B), stopcock K was closed as a precaution, since small leaks could develop from removal of the breakseal. The breakseal was subsequently removed. The volumetric and breakseal sections were now evacuated and the breakseal line checked, to ensure that no leaks had developed from careless removal or some weakness in the glass after its removal. Once completely sure of no leaks, the stopcock K was opened and the pressure recorded to ensure as perfect a vacuum as possible. The other two samples were then run in exactly the same manner.

After the removal of each CO_2 sample, the temperature of the furnace was noted. Usually after the extraction of the three specimens, the furnace was cool enough to prevent any partial reduction of newly introduced samples. Thus the combustion tubes were opened to the atmosphere gently through stopcocks O and S, after all three specimens were extracted. Once these chambers were at atmospheric pressure, the three groundglass caps (C) were removed and the plati-

num boats were then extracted. Careful removal of these boats was essential since it was necessary to reweigh them to obtain another estimate of the completeness of the reaction. This estimate, however, is only valid if most of the product of the reaction was CO_2 , but does give a good indication of the percentage CO initially in the quartz combustion tubes. That is, it can give the percentage of CO produced from the reduction of sulphate by graphite. When the reduced specimens were removed, new samples were then introduced as previously mentioned. If the contents of the old platinum boats, that is, the BaS, was to be examined for its sulphur isotope ratios, then the residue from the boats was leached with warm water and filtered into AgNO_3 , so that the resulting silver sulphide could be burned.

2.4 Standards, Correction Factors, and Reproducibility

SILVERMAN (1951) defined the zero of his isotopic scale to be oxygen from a single sample of sea water, "Hawaiian sea water No. RT6". In the published data of CLAYTON and EPSTEIN (1958) and TAYLOR and EPSTEIN (1962), the standard was defined to be the oxygen with an $\text{O}^{18}/\text{O}^{16}$ ratio equal to $0.98473 R_p$, where R_p was the $\text{O}^{18}/\text{O}^{16}$ ratio in SILVERMAN'S (1951) sample of Potsdam sandstone. If any of the analytical procedures introduce systematic errors into the measured values,

then normalization to a quartz standard should make the results of different analytical methods agree on the quartz samples. This is however not necessarily true of other minerals and rocks.

The oxygen of mean ocean water is a logical standard for the reporting of oxygen isotope variations in natural materials, including meteoric waters, rocks, and minerals. Such a standard has been proposed by CRAIG (1961) for natural waters and by CLAYTON and CRAIG (1962) for rocks and minerals. A "standard mean ocean water", that is SMOW, was defined to have a

$$O^{18}/O^{16} \text{ (SMOW)} = 1.008 O^{18}/O^{16} \text{ (NBS - 1)} ,$$

where NBS - 1 is a water sample distributed by the National Bureau of Standards. This defines an ocean water standard in terms of materials readily available to everyone, rather than in terms of the limited quantity of a particular sample of Potsdam sandstone.

All the oxygen isotope abundance measurements are expressed in parts per thousand with respect to SMOW ("standard mean ocean water") as defined by CRAIG (1961). The usual terminology is used, where δ ("del") is the deviation in parts per thousand ($^0/_{oo}$) of the O^{18}/O^{16} ratio from that of the standard. That is, the per mil isotopic ratio enrichment in a sample, relative to the standard, can be expressed as

$$\delta^{18} \text{O} \text{ (}\text{‰}\text{)} = \frac{(\text{O}^{18}/\text{O}^{16}_{\text{sample}} - \text{O}^{18}/\text{O}^{16}_{\text{std}})}{(\text{O}^{18}/\text{O}^{16}_{\text{std}})} \cdot \frac{1000}{1}$$

$$= \left(\frac{\text{O}^{18}/\text{O}^{16}_{\text{sample}}}{\text{O}^{18}/\text{O}^{16}_{\text{std}}} - 1 \right) \cdot \frac{1000}{1} .$$

CRAIG (1961) introduced the more practical SMOW standard in terms of the PDB standard, where SMOW was defined as

$$\delta^{18} \text{O}_{\text{sample}} = 1.0409 \delta^{18} \text{O}_{\text{sample}} - 40.92 \text{ ‰} .$$

wrt SMOW

wrt PDB

The PDB scale referred to the $\text{O}^{18}/\text{O}^{16}$ ratio in the CO_2 gas evolved from the reaction of H_3PO_4 with PDB carbonate. This Chicago standard for CO_2 was produced from PDB calcium carbonate by reaction with 100 per cent H_3PO_4 at 25.2°C . (The PDB was a Cretaceous belemnite, from the Pee Dee formation of South Carolina.)

CRAIG (1957) derived correction factors applicable in the conversion of ion abundance ratio differences to the specific isotope ratio differences. These correction factors, for the mass spectrometric analysis of CO_2 , arise because for the analysis of oxygen we measure the mass - 46 beam versus

the combined mass - 44 plus mass - 45 beam. Hence the ratio in terms of the isotopic molecules is:

$$\frac{C^{12}O^{16}O^{18} + C^{13}O^{16}O^{17} + C^{12}O^{17}O^{17}}{C^{12}O^{16}O^{16} + C^{13}O^{16}O^{16} + C^{12}O^{16}O^{17}}$$

whereas the desired ratio, assuming the distribution to be purely a statistical one, is:

$$\frac{C^{12}O^{16}O^{18} + C^{13}O^{16}O^{18}}{C^{12}O^{16}O^{16} + C^{13}O^{16}O^{16}} = \frac{C^{12}O^{16}O^{18}}{C^{12}O^{16}O^{16}}$$

As a result, the measured isotopic ratio for oxygen must be corrected for the other isotopic species. The correction must be such that

$$\delta O^{18} = 1.0014 \delta O^{18} - 0.009 \delta C^{13},$$

which is the correction factor used for oxygen analysis made against the PDB standard. Thus, about 1 per cent of the δC^{13} difference enters into the oxygen correction factor.

Using CRAIG'S (1957) correction factor as well as CRAIG'S (1961) "standard mean ocean water" (SMOW) reference, the isotopic reproducibility was examined for the reduction of sulphate by graphite, yielding CO_2 as the final gaseous product. The volume of oxygen that was recovered as CO_2 was always

greater than 94 per cent of the theoretical value, hence BaSO_4 was considered to be quantitatively reduced. Any CO formed in the reduction process was completely converted to CO_2 ; this was ascertained by the volumetric check on reaction completeness.

It was then necessary to check the isotopic reproducibility. A sample of sea water sulphate was selected for the reproducibility tests. These results yielded an average δO^{18} value of $+9.38 \text{ }^{\circ}/\text{oo}$ with respect to SMOW (Table 2-2). The precision during a mass spectrometric analysis was typically $\pm 0.05 \text{ }^{\circ}/\text{oo}$, while the reproducibility over preparations of the same specimen was $\pm 0.12 \text{ }^{\circ}/\text{oo}$ (the standard deviation). The preparation and reduction reproducibility were better than the $\pm 0.18 \text{ }^{\circ}/\text{oo}$ achieved by RAFTER and MIZUTANI (1967a). Thus the techniques employed in the present investigation were considered to be satisfactory for studies of oxygen isotope abundance variations in sulphate.

2.5 Investigations on the Graphite Reduction of Sulphate

The graphite reduction of sulphate was considered quantitative and reproducible when the procedures previously outlined were employed. Other workers also achieved reasonable reproducibility but with a wide variety of techniques. These investigators seemed to have their own biases

Table 2-2 Oxygen isotopic composition
of sea water sulphate

Specimen Number	Weight of BaSO ₄ (mg)	CO ₂ Volume (cc at NTP) Theo. Exp.		$\delta \text{O}^{18} (\text{SO}_4^{=})$ ($^{\circ}/\text{oo}$) wrt SMOW	Deviation From + 9.38 AVG. ($^{\circ}/\text{oo}$)
WOS 2000	55	10.5	10.2	9.16 ± 0.02	-0.22
WOS 2001	44	8.4	8.3	9.34 ± 0.09	-0.04
WOS 2003	67	12.8	12.3	9.43 ± 0.07	0.05
WOS 2004	64	12.2	12.1	9.42 ± 0.07	0.04
WOS 2007	66	12.6	12.5	9.38 ± 0.12	0.0
WOS 2008	49	9.4	9.5	9.31 ± 0.03	-0.07
WOS 2009	56	10.7	10.6	9.38 ± 0.01	0.0
WOS 2011	51	9.7	9.6	9.46 ± 0.06	0.08
WOS 2012	43	8.2	8.3	9.17 ± 0.03	-0.21
WOS 2013	38	7.2	6.9	9.27 ± 0.05	-0.11
WOS 2015	57	10.9	10.7	9.53 ± 0.06	0.15
WOS 2017	49	9.4	9.0	9.52 ± 0.08	0.14
WOS 2018	70	13.4	12.7	9.53 ± 0.04	0.15
WOS 2020	44	8.4	8.2	9.39 ± 0.05	0.01
WOS 2021	72	13.7	13.0	9.46 ± 0.02	0.08

9.38 0.05 * ± 0.12 **

Mean $\delta \text{O}^{18} = 9.38^{\circ}/\text{oo}$

* Mean of measurement errors = $\pm 0.05^{\circ}/\text{oo}$

** Overall deviation (preparation reproducibility
and mass spectrometric error) = $\pm 0.12^{\circ}/\text{oo}$

as to the choice of crucibles, CO-CO₂ conversion units, and C/S ratios. There appeared to be no logical basis for these choices.

For the present investigation it was considered worthwhile to study the effects of varying the C/S ratio, that is, the ratio of the amount of graphite C (mg) to that of sulphate S (BaSO₄ in mg)

$$\frac{C}{S} = \frac{\text{mg of graphite before mixing}}{\text{mg of BaSO}_4 \text{ before mixing}},$$

since similar studies used markedly different graphite to sulphate ratios (section 2.1). The 15 samples used for the reproducibility study (Table 2-2) all had a C/S ratio of about 1.0, as suggested by RAFTER (1967). On the other hand, nine specimens not listed in Table 2-2 had radically different oxygen isotope compositions. In these samples, the C/S ratio was varied from 0.64 to 3.25. The oxygen isotopic abundance ratios of these variant nine sea water sulphate samples are summarized in Table 2-3 and Figure 2.2. Since all other variables remained constant, the data of Figure 2-2 seemed to imply that the C/S ratio had a significant effect upon the isotopic composition of the CO₂ produced by the reduction of sulphate.

Table 2-3 Oxygen isotope composition of nine
variant sea water sulphates

<u>Specimen Number</u>	<u>Weight BaSO₄ (mg)</u>	<u>C/S Ratio</u>	<u>δ O¹⁸ (SO₄⁼) (⁰/oo) wrt S.M.O.W.</u>		<u>Dev From +9.37 (⁰/oo)</u>
WOS - 2002	56	1.00	10.85	0.07	1.48
WOS - 2005	70	1.00	10.89	0.07	1.52
WOS - 2006	49	1.00	10.91	0.09	1.54
WOS - 2010	77	0.64	8.79	0.08	-0.58
WOS - 2014	52	1.54	10.82	0.03	1.45
WOS - 2016	43	1.96	11.53	0.07	2.16
WOS - 2019	28	3.25	10.02	0.08	0.65
WOS - 2022	44	1.05	9.96	0.03	0.59
WOS - 2023	32	1.09	10.60	0.07	1.23

(NOTE: Samples WOS - 2002, 2005, and 2006 were only evolved carbon dioxide. The carbon monoxide produced in these cases was pumped away to permit a determination of the isotopic composition of the CO₂ product only.)

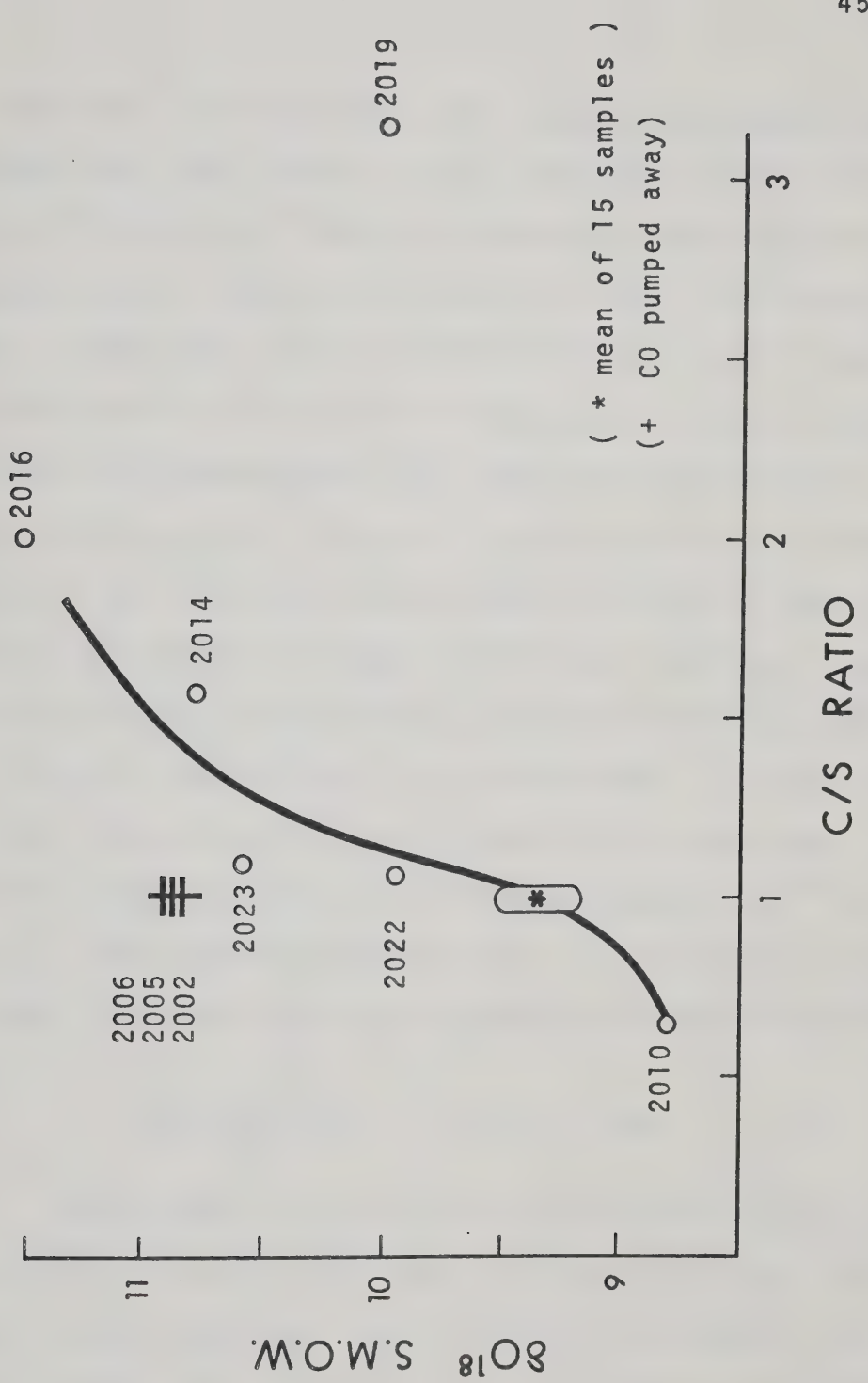


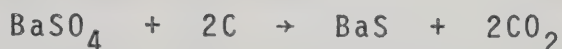
Figure 2-2 Oxygen isotopic composition of nine variant sea water sulphates.

with graphite. It should be noted that three results were not anomalous, since after the CO_2 condensed in the cold traps, the CO product formed at the end of the reduction was pumped away. These three samples, WOS - 2002, WOS - 2005, and WOS - 2006, all had a C/S ratio of about 1.0. These three specimens, used to obtain the oxygen isotope abundance ratio of the evolved CO_2 , neglected the CO which formed simultaneously. The δO^{18} value of the CO_2 produced was higher than expected, which means that the CO was enriched in the lighter isotope relative to the CO_2 . It was only when the CO was sparked and converted into CO_2 that the lower value of about $+9.38 \text{ }^0/\text{oo}$ was reached. It should be noted that the collected CO_2 in these three cases was heavier by about $1.5 \text{ }^0/\text{oo}$ (the CO consequently $1.5 \text{ }^0/\text{oo}$ lighter). With approximately 94 per cent recovery, sparking would have left the remaining unreacted CO heavier by about

$$\frac{94 (1.5 \text{ }^0/\text{oo})}{6} = + 23.5 \text{ }^0/\text{oo} .$$

Thus three of the variant nine samples were processed so as to obtain the oxygen isotope abundance ratio of only the CO_2 produced, ignoring any contribution from the CO evolved. The remaining six were not in any manner designed to be anomalous or at variance with the aims of the reproducibility study. Hence a closer look at these six was mandatory.

Any explanation for such widely varying isotopic abundance ratios of the sea water sulphate samples must consider the relative proportions of carbon and sulphate mixed prior to the reduction process. The C/S ratio was usually kept about 1.0 only as a result of RAFTER's (1967) implication. This relative proportion was in fact stoichiometrically more than sufficient, since the reaction

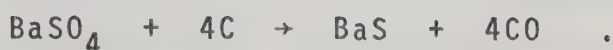


required only the ratio described in the above equation. In such a case the C/S ratio stoichiometrically necessary was simply

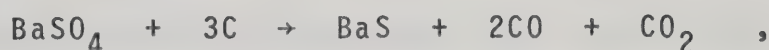
$$\frac{2\text{C}}{\text{BaSO}_4} = \frac{2(12)}{137 - 96} = \frac{24}{233} = 0.10 \quad .$$

That is, stoichiometrically the reaction should have proceeded to completion if there was approximately one part graphite for every ten parts BaSO_4 (by weight). Any C/S ratio greater than 0.1 completely satisfied the stoichiometric requirements. The question then arose, why were variations of the C/S ratio causing the observed discrepancies in the oxygen isotope abundance ratios, considering the fact that this was about 10 times the stoichiometric ratio required? Also, was this then a reaction where stoichiometry was applicable?

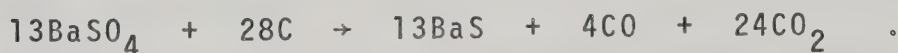
Before making hasty conclusions concerning the requirements of the primary product of the reduction, it was necessary to also consider the second gaseous product of this reduction. Most probably the CO formed such that



In this case the C/S ratio would be about 0.20. For some combination of the two reactions, which was the observed result for such a reduction of sulphate by graphite, then the reaction could have been of the form



balanced for primarily CO evolution. In the present study, the relative amounts of gaseous product were in the order of about 15 per cent CO and about 85 per cent CO₂. This reaction was approximately



Stoichiometrically, the C/S ratio would be only 0.07. That is,

$$\frac{28\text{C}}{13\text{BaSO}_4} = \frac{14(2\text{C})}{19(\text{BaSO}_4)} = \frac{14}{19} (0.10) = 0.07 .$$

The requirements were relatively unchanged from the previous estimate in which the gaseous final product was just carbon dioxide.

Thus, stoichiometric considerations and the known products of the reduction did not seem to explain the experimental isotopic ratio differences. The only conclusion left, was that the reaction proceeded to near completion (that is, greater than 94 per cent reaction) without doing so stoichiometrically. This implied some form of mechanical parameters, such as a dependence upon the availability of nearby atoms of graphite. This involved physical contact availability, since the mixing was only a mechanical process, and definitely not a chemical procedure. Here the reduction could have possibly been controlled by the presence or absence of contact carbon atoms which would permit the removal of oxygen atoms from barium sulphate. Neither pellets nor compression of the sulphate and carbon sample, to increase the physical availability of graphite, were investigated. Such procedures would have ensured good physical contact and most probably would have decreased the C/S ratio required to cause a quantitative reduction. Hence compression of the graphite and sulphate after mixing, and the degree of compression, could have altered the C/S ratio necessary to cause the observed variations in the oxygen

abundance ratios.

If mechanical mixing caused the discrepancies recorded in Table 2-3, then an excess of contact graphite was expected to increase the amount of CO formed relative to the CO₂ gaseous product. This interpretation was based on the C/S ratios calculated to yield either a total CO or CO₂ final product. A C/S ratio of 0.10 was found to be stoichiometrically sufficient for a CO₂ final product, whereas a ratio of 0.20 was required for CO to be the final product. Thus stoichiometrically, more graphite was required to produce a final product of CO than CO₂. Although the reduction did not proceed in a totally stoichiometric fashion, the C/S ratio implications were believed applicable for the mechanical mixing of graphite and sulphate. In other words, the graphite reduction of sulphate was both mechanical and stoichiometric in nature, with possibly physical contact the most dominant factor. As such, graphite excesses were expected to yield greater quantities of CO. This was confirmed by observation of the sparking time as well as by evaluation of the per cent CO product. This per cent CO formation was calculated using the known weight of the platinum boat and its contents after the reaction had gone to completion. For example,

Sample WOS - 2010	boat plus lid	= 1393.2 mg
	BaSO ₄	= 77.1 mg
	C	= 48.5 mg
	C/S	= 0.65
	total weight before	= 1518.8 mg
	after reduction	= 1489.5 mg
	reactants	= 29.5 mg

available oxygen for reduction = 77.1 mg ($\frac{64}{233}$) = 21.16 mg

carbon stoichiometrically required = 21.16 mg ($\frac{24}{64}$) = 7.94 mg

stoichiometric reactants = 29.10 mg

The measured reactants weighed 29.5 mg while the stoichiometric requirements were only 29.1 mg. Hence 0.4 mg of extra carbon was used. Since the total graphite used was 8.34 mg, then the

$$\text{CO per cent production} = \frac{(8.34 - 7.94)}{(8.34)} (100 \%) = 4.8 \%$$

Therefore per cent CO formed was about 5 % .

With an excess of graphite available for the reduction of sulphate, a marked increase in CO production was observed (Table 2-4). If a C/S ratio of about 1.0 was employed, the per cent CO was normally in the order of 15 per cent whereas the CO₂ production was about 85 per cent of the

Table 2-4 Per cent CO production for sea water sulphates reduced with graphite

<u>Specimen Number</u>	<u>C/S Ratio</u>	<u>Dev. From +9.38 ⁰/100</u>	<u>Per Cent Prod.</u>	<u>Per Cent Reaction</u>
WOS - 2010	0.64	-0.59	5	94
WOS - 2000	0.91	-0.22	8	97
WOS - 2001	1.00	-0.04	13	99
WOS - 2003	1.00	0.05	9	96
WOS - 2004	1.00	0.04	12	99
WOS - 2007	1.00	0.0	21	99
WOS - 2008	1.00	-0.07	14	101
WOS - 2009	1.00	0.0	12	99
WOS - 2011	1.00	0.08	16	99
WOS - 2012	1.00	-0.21	10	101
WOS - 2013	1.00	-0.11	15	96
WOS - 2015	1.00	0.15	23	98
WOS - 2017	1.00	0.14	10	96
WOS - 2018	1.03	0.15	19	95
WOS - 2020	1.03	0.01	15	98
WOS - 2021	1.03	0.08	29	95
WOS - 2022	1.05	0.58	27	97
WOS - 2023	1.09	1.22	49	95
WOS - 2014	1.54	1.44	42	95
WOS - 2016	1.96	2.15	46	94
WOS - 2019	3.25	0.64	63	94

final gaseous product. However, when C/S ratios removed from 1.0 were employed, the relative amounts of CO and CO₂ were much different. The effects of varying the C/S ratio can be seen more clearly when the data of Table 2-4 is plotted as in Figures 2-2 and 2-3. Generally there was an increase in δO^{18} and per cent CO production with increasing C/S ratios (Figures 2-2 and 2-3 respectively). These observations were somewhat dependent upon the per cent reaction obtained during the reduction process (Figure 2-4), when C/S ratios other than 1.0 were employed.

It was demonstrated that if the C/S ratio was greater than 1.0, more than 15 per cent of the gaseous product was CO, with increasing amounts of CO as the C/S ratio rose. Since more CO was present, more CO \rightarrow CO₂ conversion was required. It was observed that since lengthy conversions often yielded results which were not quantitative. The variant samples all had relatively low yields (about 94 per cent reaction), and generally large amounts of CO were formed. Although the reductions were quantitative, the O¹⁸/O¹⁶ isotopic ratios did reflect the CO abundance caused by the larger C/S ratios investigated in this study.

More reductions were carried out at C/S ratios less than 1.0, but the majority were not quantitative. (It should be noted that no other investigation ever employed

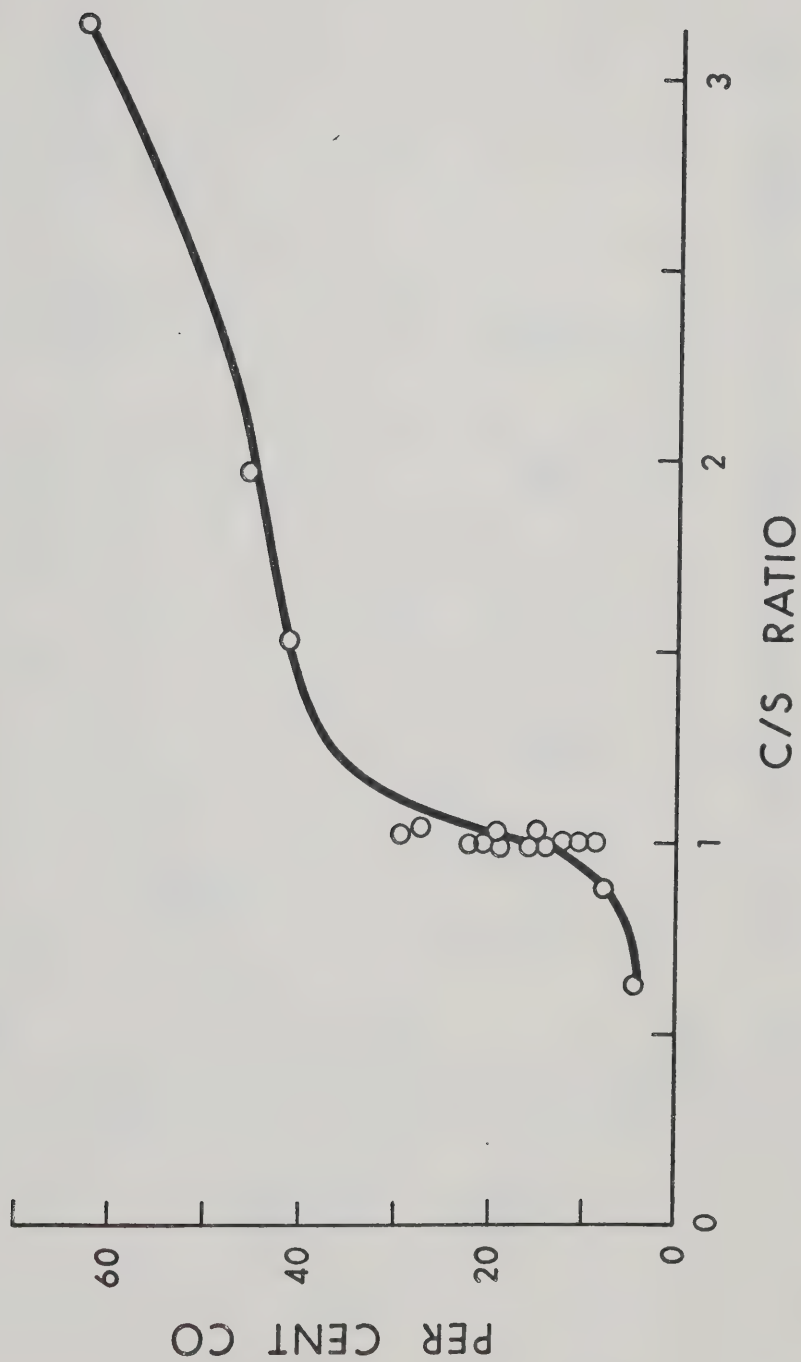


Figure 2-3 Per cent CO production and the c/s ratio.

a C/S ratio less than unity.) A deficiency of contact graphite atoms was believed to be an explanation for the poor yields. Incorporation of further mixing while the reaction proceeded, or compression of the graphite and sulphate sample before the reduction process, would probably have reduced the numerical value of the optimum C/S ratio, but not the variations in CO formation.

RAFTER (1967) stated that the graphite reduction of sulphate occurred within the temperature range 900 to 1050°C, and observed that graphite reduction was measurable at 960°C. Longinelli (private communication) found that 50 per cent of his CO₂ had been produced at 900°C. Therefore it was concluded that the reduction of sulphate by carbon was well under way by about 900°C. In such a reduction where CO evolution has been shown to be of utmost importance, it was decided to monitor the CO formation as a function of the reduction temperature.

The vacuum gauge used throughout the reduction process, was employed to measure pressure variations as the reaction proceeded. As the reduction began, the decrease in pressure was in reality a measure of gas evolution during the reduction of the sulphate. The evolved gas was considered to be totally CO, since any CO₂ formed was immediately condensed as soon as it was produced. Therefore the vacuum

readings evaluated the qualitative amounts of CO formed.

In those cases where the C/S ratio was such that there was not sufficient contact graphite to ensure that each BaSO_4 molecule was totally stripped of its oxygen atoms, the possibility of other gas evolutions should not be ignored. In addition to CO and CO_2 formation, undesirable products such as SO_2 and some CSO derivatives may have been present. These were for the most part trace products, since the reactions presented (Table 2-4) were all quantitative. Although these undesirable gases were most probably present, they were ignored when considering the recorded pressure readings as evidence of CO formation.

The production of CO was monitored as the reduction temperature rose, for varying C/S ratios. The quality of this data was limited by the vacuum gauge utilized. The results are shown in Figure 2-5. It was felt that the C/S ratio should dictate the production rate of CO. Figure 2-5 suggested that below 960°C , the C/S ratio did not influence the CO production rate since the curves are similar for 5 different C/S ratios varying from 0.63 to 1.86. The BaSO_4 reduction, as noted by RAFTER (1967), was well under way by about 960°C . Behavior above this temperature was difficult to monitor because of the large amounts of CO evolved, and the inaccuracy of the meter in this pressure range. It does

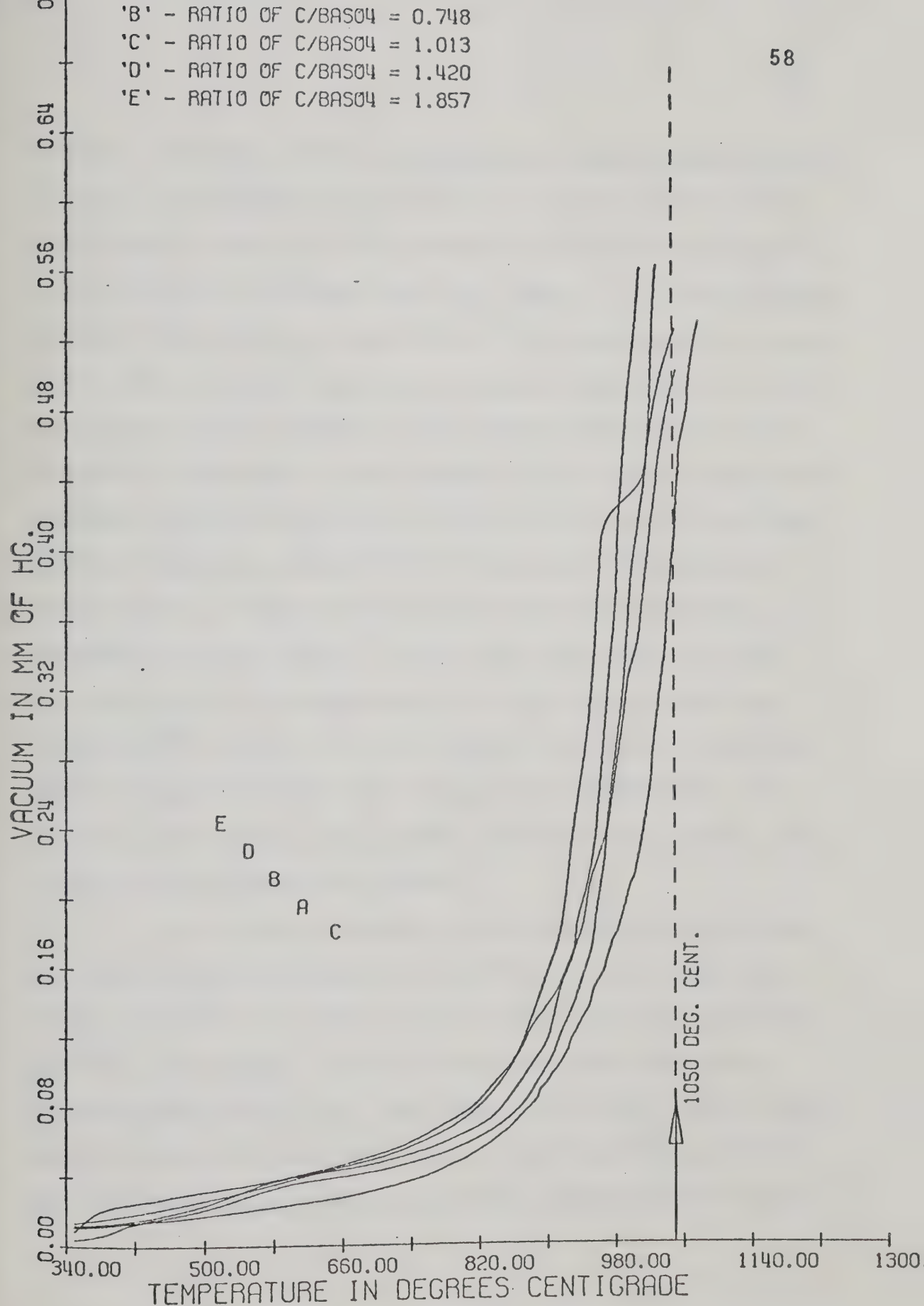


Figure 2-5 CO production and the reduction temperature.

follow, that most of the CO production occurred above 960°C.

It should be noted that the samples with a C/S ratio greater than the experimental optimum of 1.0 always indicated more CO present than the others. This was seen to be true from the very beginning of the reductions in Figure 2-5. Even at lower temperatures, where appreciable CO formation was not in evidence, the relative amounts of gas evolution suggested a trend towards more CO when there were excesses graphite available. With a deficiency of optimum contact graphite, production of the undesirable gaseous forms may be an interpretation for the higher pressures recorded in these cases. It was also concluded that outgassing and drying temperatures for the sample must not exceed 500°C. Here a distinct evolution of CO was present. Such a temperature was never used, nor recommended, especially if excesses of graphite were more than about 8 or 9 times the stoichiometric demand.

The relative amounts of CO and CO₂ evolved, were not as constant as expected. RAFTER's (1967) study found CO-CO₂ mixtures to contain about 75 per cent CO₂ and 16 per cent CO. His study, as previously mentioned, utilized platinum boats and lids. LONGINELLI and CRAIG (1967) found their mixtures to vary from about 98 per cent CO₂, with a new crucible, down to 80 per cent CO₂ after about 10 reac-

tions with their graphite containers. The mixture of the present study usually was in the order of 85 per cent CO_2 with about 15 per cent CO when the C/S was optimized. With the lower C/S ratios, about 90 per cent of the gaseous product was CO_2 , whereas, the higher C/S ratios yielded only about 55 per cent CO_2 .

Further mixing of the carbon and sulphate was an impossibility. Compression of the sample could have, as earlier noted, permitted a much reduced C/S ratio, and hence less CO formation. LLOYD (1968) ground his sulphate sample with a tenfold excess of graphite, forming the mixture into a pellet (no information was given on the degree of compression used). From the results of the present study, it was concluded that the most quantitative and reproducible results can only be obtained by sample compression, C/S ratio optimization, and platinum containers.

It is clear that by working with a C/S ratio of about 1.0, very good reproducibility was obtained as previously found by RAFTER (1967). It may be that if many samples had been run at another C/S ratio, the reproducibility would have been acceptable - although the δO^{18} value may have been different. No doubt further work could have elucidated this phenomenon. It was decided to pursue microbiological isotope fractionation studies using a C/S

ratio of 1.0. This choice was based on the reproducibility obtained with this ratio, and a desire not to deviate significantly from conditions utilized in other laboratories.

It is interesting to note that during the present study, SAKAI and KROUSE (1971) looked at various aspects of the BaSO_4 reduction. They found that during the conversion of CO to CO_2 , CO^{16} reacted about 1.02 times faster than CO^{18} . (This is in agreement with the data of the present study where the CO formed was pumped away.)

SAKAI and KROUSE (1971) investigated the graphite reduction of sulphate in an attempt to explain why sulphates of widely varying oxygen isotopic compositions were not reproducible. When such samples were analyzed, the isotopic precision deteriorated to greater than ± 0.5 ‰, despite satisfactory yields. The possibility of memory effects was investigated. The repeated conversion of samples from the same BaSO_4 source, where BaSO_4 with significantly differing δ ‰ values had been previously processed, resulted in a very large memory phenomena. With an externally heated quartz tube, these effects were recorded in the order of 2.5 ‰. Also a tube displaying signs of devitrification after prolonged usage produced larger memory phenomena than relatively new quartz tubes. In fact even new tubes displayed some memory effect, but this was logically related to its process of manufacture.

A possible explanation of this memory phenomena was that pure adsorption had occurred, the product gases being retained by the quartz walls. As pointed out by SAKAI and KROUSE (1971), this was not possible since the yields were consistently greater than 95 per cent. They concluded rather that an exchange had occurred, since the reductions were quantitative. Such an exchange of oxygen atoms was suggested between the hot quartz walls and the gaseous products. It was further noted that NORTHRUP and CLAYTON (1965) reported oxygen isotope exchange between CO_2 and glass at temperatures above 250°C . However any CO_2 present should not have been a participant in such an exchange phenomena, because during the reduction of sulphate, it was condensed as soon as it was formed. Any CO, however, would have remained in the gaseous phase until converted by the high voltage discharge, thus having a much better opportunity to exchange oxygen isotopes with the hot quartz walls. (Such a phenomenon would have been much more pronounced, had there been larger percentage yields of carbon monoxide.)

Furthermore SAKAI and KROUSE (1971) noted the relative importance of CO formation. The amount of CO which remains at the end of the heating cycle, they stated, was just a fraction of the CO actually produced during the

earlier stages of the reaction. This was based on six experiments, where it was found that the CO collected during the reduction process corresponded to as much as 50 per cent of the total oxygen present. Thus in certain stages of the conversion, sufficient CO was available to have participated in the oxygen exchange with the hot quartz walls, thereby producing the noted memory effect.

Such excessive amounts of CO evolved were most probably due to an excessive C/S ratio. That is, there was clearly an excessive amount of graphite present otherwise there would not have been such a large amount of CO formed. No compression technique was employed in their study. However the CO formed must have been subsequently oxidized to CO₂, since the actual and the theoretical yields were consistent.

- This memory effect was not applicable to the present reproducibility study, since the BaSO₄ samples used were obtained from the same barrel of sea water. When sulphate was used with varying oxygen isotope compositions, inconsistencies were noted and any such samples were repeatedly reduced until the isotopic reproducibility was considered satisfactory. This was done by successively converting a given sample in the same quartz until the δO^{18} values were consistent with the desired reproducibility.

Normally, two reductions, in succession in the same quartz tube, were carried out for each isotopic determination.

Although duplication of analyses should always be an integral part of the isotope abundance ratio determinations, often in cases where the material is rare, only one measurement is possible. To avoid the memory phenomenon, the best solution is to eliminate the memory effect completely. In the present study, such a policy was not available. SAKAI and KROUSE (1971) reduced this memory effect by utilizing the electrical discharge unit throughout the reduction. This permitted the continual removal of CO as the reduction proceeded (this was done as routine procedure in the present study). Although they noted a reduction in the memory phenomena, it was not entirely removed by such a continuous CO conversion. The only solution lay in a more efficient CO-CO₂ conversion unit. LLOYD (1967) and LONGINELLI and CRAIG (1967) used induction heating, which was a possible solution. SAKAI and KROUSE (1971) preferred to use an internally heated apparatus for the carbon reduction, but decided to cool the quartz tube walls, thus minimizing the oxygen exchange. A platinum boat was made to be the location of highest resistance in an electrical circuit, thus avoiding external heating. The quartz walls were retained, but became the inner part of a water jacket around the platinum boat.

Thus the quartz walls were kept cool by the water jacket, and eliminated any possibility of oxygen isotope exchange between the CO evolved and the quartz tube. Their apparatus even showed no signs of memory effects when samples differing in δO^{18} values by over 22 ‰ were analyzed. The present study did not have the advantage of such an apparatus, consequently when varying isotopic composition samples were run, they were done successively in the same quartz combustion tube. Duplication showed up the memory effect, but such widely varying specimens were not a part of the present investigation.

2.6 Summary

1. A method has been developed by RAFTER (1967) for the recovery of oxygen from sulphate for the study of oxygen isotope variations in sulphates.

2. The reproducibility of this method is at present ± 0.1 parts per mil, when the C/S ratio is optimized. Further optimization might be obtained by; (a) compression of the sulphate and graphite sample into a pellet before the reduction, (b) mixing during the reduction process, and (c) the use of platinum boats and lids.

3. This method permits both sulphur and oxygen isotope abundance variations in sulphate on the same sulphate specimen.

4. The graphite to barium sulphate ratio, by weight, does affect the carbon monoxide percentage evolution. The δO^{18} values generally increased with an increasing C/S ratio, as did the CO production.

5. The carbon monoxide formed must be completely converted to carbon dioxide, since it contains a significant portion of the oxygen atoms from the sulphate.

6. Excess graphite was considered necessary for the complete reduction of sulphate. Physical contact might dictate the percentage reaction as well as the relative amount of carbon monoxide formed.

7. Graphite boats produce variable amounts of carbon monoxide gas, depending upon the age of the containers. Any inherent errors may easily be avoided by the employment of platinum for the container metal.

8. Outgassing and drying temperatures for the sulphate specimen must not exceed 500°C . At such a temperature, distinct carbon monoxide evolution will occur.

9. Carbon dioxide gaseous product is formed below 900°C , whereas carbon monoxide evolution does not occur until about 960°C .

10. Memory effects, due to an exchange phenomenon between CO and the quartz walls, can be successfully eliminated by employing a water jacket as outlined by SAKAI and KROUSE (1971).

11. It may be that differing C/S ratios will yield reproducible and quantitative results, although the δO^{18} value may be somewhat different.

CHAPTER III MASS SPECTROMETRY

3.1 Introduction

With the development of a reproducible and reliable method for the extraction of oxygen atoms from sulphate for precise isotopic analysis, it became necessary to scrutinize the mass spectrometric precision encountered during routine stable isotope measurements.

Once the isotopic reproducibility during the reduction of sulphate by graphite reached about ± 0.12 ‰, it was realized that the precision of the isotopic measurements was typically of the same magnitude (± 0.1 ‰). Any improvement in the sample preparation reproducibility would have been futile with a mass spectrometric accuracy less than that of the chemical techniques. A possible remedy was concluded to be the recording and evaluation of more mass spectrometric isotopic ratios. Such a solution required more time and labor since a greater number of measurements had to be evaluated. In addition, the routine fluctuations of the source region and the gas pressure would have caused greater variability within a particular analysis if lengthier analyses were performed.

These problems were partially overcome by replacing the original Hewlett-Packard voltmeter and voltage to frequency converter by a Fluke Digital Voltmeter. The digitizing interval

for the Fluke equipment was 18 m sec compared to 1 sec with the Hewlett-Packard integrating voltmeter. With this faster response time, it was decided to couple the Fluke Digital Voltmeter with a computer that could handle large amounts of data in a very short time. This combination was believed to yield a slight increase in the mass spectrometric precision, typically to ± 0.05 ‰, as well as greatly increasing the convenience of tabulating the isotopic abundance ratio measurements. The actual application of the PDP-8 computer will be discussed in Section 3.3 .

3.2 The Mass Spectrometer

The mass spectrometer employed for the isotopic abundance measurements was a 90° , 12 inch radius magnetic analyzer (NIER (1947), McKINNEY, McCREA, EPSTEIN, ALLEN, and UREY (1950)). The O^{18}/O^{16} abundance ratios in sulphates were measured by comparing mass 46 ($C^{12}O^{16}O^{18}$) to mass 44 ($C^{12}O^{16}O^{16}$). The corresponding S^{34}/S^{32} isotopic data was obtained from masses 66 ($S^{34}O^{16}O^{16}$) and 64 ($S^{32}O^{16}O^{16}$).

In the determination of isotopic compositions, single collection often realizes precisions of about 0.1 per cent. The limitation arises primarily from real time fluctuations in the mass spectrometer source region. Such a limitation is usually eliminated by simultaneously collecting ion currents as described by STRAUS (1941), and later modified by numerous workers. Thus the isotopic ratio of a standard and an unknown

sample can be compared improving the mass spectrometric precision to about 0.01 per cent within an analysis.

The standard and unknown gases were introduced into the mass spectrometer through small leaks, designed to reduce the pressure. Any back pressure on these leaks was maintained at a constant level by adjusting the mercury level of the gas reservoirs in the introduction sector. Four magnetic valves were employed to permit alternate measurement of the standard and unknown gas sample ion current. The gas (CO_2 or SO_2) was then ionized and accelerated by a potential difference of about 4 kv into the magnetic field. Ion currents corresponding to either masses 44 and 46 for CO_2 , or 64 and 66 for SO_2 , were collected in Faraday cups and originally amplified by a pair of Cary vibrating reed electrometers. The output voltage from one of the vibrating reed electrometers was converted to frequency and used as the time base reference for the Hewlett-Packard voltmeter as described by McCULLOUGH and KROUSE (1965), thus the voltmeter and frequency converter combination was employed as a ratio measuring device. The pressures of the standard and unknown sample as well as the peak shape was monitored by a two pen chart recorder.

This method has since been replaced by a Fluke Digital Voltmeter, as mentioned earlier, with a ratio option. This system was further modified by the interfacing of a

PDP-8 computer to the Fluke voltmeter in order to eliminate tedious data reduction.

3.3 PDP 8 Data Handling

With the rapid influx of data from the faster response Fluke equipment, the precision of mass spectrometric determinations was observed to increase slightly, because of the greater flexibility in averaging time that became available. Thus the introduction of the PDP-8 computer involved integration of the data. It was decided to program the PDP-8 such that it would also evaluate the mean isotopic ratio, as well as the standard deviation obtained during that analysis.

Two samples of the computer output are shown in Figure 3-1 and Figure 3-2. The program was initiated by the operator typing one line of comments. This provided the necessary identification and other pertinent information of sample or standard. Once this line was completed and the RETURN key depressed, the program would type the message $INTIME = 2^{**}(\underline{m})$, expecting some single digit to be the reply. This reply was an indication of how many recordings constituted such sub-set mean. That is, the digit typed was the exponent to which the base 2 was raised to ascertain how many values were to be taken. Normally 7 was typed for INTIME, where 2^7

INTIME=2**7

GMTIME=2**2

FIRST SET MUST BE S !

THIS SET IS S

0.41945 0.41948 0.41954 0.41947

0.41949 S

THIS SET IS X

0.42045 0.42041 0.42044 0.42048

0.42045 X

THIS SET IS S

0.41967 0.41073 0.41969 0.41968

0.41969 S

THIS SET IS X

0.42057 0.42071 0.42053 0.42064

0.42061 X

THIS SET IS S

0.41979 0.41978 0.41977 0.41977

0.41978 S

THIS SET IS X

0.42072 0.42063 0.42066 0.42069

0.42068 X

THIS SET IS S

0.41989 0.41989 0.41987 0.41986

0.41988 S

THIS SET IS X

0.42081 0.42075 0.42126 0.42044

0.42082 X

1.00204 1.00200 1.00208 1.00206 1.00202 1.00207

MEAN RATIO 1.00204

STD DEV IS 0.00003

TYPE Y IF DEV SATISFACTORY? Y

4:45 PM , 28 / 2 / 72 . SC3 AS X , SAME S .

INTIME=2**7

GMTIME=2**2

FIRST SET MUST BE S !

Figure 3-1 Typical PDP-8 output.

THIS SET IS S

0.41824 0.41809 0.41814 0.41821

0.41817 S

THIS SET IS X

0.41641 0.41638 0.41611 0.41633

0.41631 X

THIS SET IS S

0.41877 0.41871 0.41881 0.41892

0.41880 S

THIS SET IS X

0.41683 0.41690 0.41687 0.41682

0.41686 X

THIS SET IS S

0.41908 0.41917 0.41913 0.41923

0.41915 S

THIS SET IS X

0.41683 0.41712 0.41690 0.41687

0.41693 X

THIS SET IS S

0.41904 0.41917 0.41919 0.41910

0.41913 S

THIS SET IS X

0.41692 0.41696 0.41695 0.41695

0.41695 X

0.99480 0.99471 0.99495 0.99462 0.99472 0.99477

MEAN RATIO 0.99476

STD DEV IS 0.00011

TYPE Y IF DEV SATISFACTORY?

D

DELETE 4

DELETE N

MEAN RATIO 0.99479

STD DEV IS 0.0010

TYPE Y IF DEV SATISFACTORY?

D

DELETE 3

DELETE N

MEAN RATIO 0.99475

STD DEV IS 0.00004

TYPE Y IF DEV SATISFACTORY?

Y

readings were algebraically averaged from the Fluke to constitute a sub-set mean. This provided an average over approximately 3 seconds.

The $GMTIME = 2^{**}(\underline{n})$ requested, was a demand for the number of such sub-sets to be evaluated before the final calculation of the ion current ratio for that particular gas sample. Each final ratio was hence the mean of 2^n sub-sets of 2^m intervals. This initialization procedure became obligatory for the remainder of the S (standard) and X (unknown) sample determinations. These symbols were also typed in, so as to remind the operator of which sample was to come next. If for some reason it was decided to start over without completing the predetermined number of analyses, then an H (halt) was typed instead of the S or X.

Four isotopic abundance ratios were evaluated for both the standard and unknown gas samples. The final X/S ratio was then calculated by taking the average value of the first two S ratios and dividing this into the first X value. The average of the first two X values was divided by the second value of the S ratio, and so on for the eight S and X ratios. This resulted in six values for X/S which were then averaged. The result of which was printed following the message MEAN RATIO. In addition, the standard deviation was evaluated and presented below the mean ratio. This standard deviation calculation was not rigorous, but rather simply an estimate of the mass spectrometric stability.

If the standard deviation was considered satisfactory, then when prompted by the computer operator who typed Y on the teletype, the program was ready for a new analysis beginning with one line of comments. If however the standard deviation was not considered to be adequate, due to some operator error in running the mass spectrometer or instrumental instabilities, the typing of a D instead would enable the delete subroutine. Thus any of the X/S ratios could be deleted.* The appropriate ratio was then ignored from a recalculation of the final X/S ratio and the resultant standard deviation. A standard deviation of under ± 0.1 ‰ was considered acceptable, although routinely they were about ± 0.05 ‰ if the INTIME and GMTIME were sufficiently large (m about 7 and n as 2).

The actual program permitted the options mentioned while rejecting erroneous demands or mistakes made by the operator. The software was written in assembler (PAL III) language because of the limited storage available in such a small computer and a listing may be found in Appendix A.

The convenience and precision offered by this program was a definite asset during isotopic abundance ratio studies, and has greatly reduced the monotony of such analyses. The ease of data handling was considered sufficient grounds for the writing of an expanded program which would operate three

(* The delete facility is designed to permit calculation of the data after some malfunction. It is to be used sparingly.)

mass spectrometers in a similar manner.

This enlarged time-sharing program was written and tested successfully for the simultaneous operation of three mass spectrometers. The length alone prohibits incorporation of the program into this thesis.

It was envisaged that the PDP-8 and its time-sharing system could successfully be applied to the maintenance of good peak shape, the alteration between the standard and unknown, as well as for control of standard and unknown gas pressures. Such a scheme seemed beyond the scope of a simple PDP-8 time-sharing system. As a result, a T. I. 980 A computer has since been employed to supercede the PDP-8. Although the programming is still in progress, it is obvious that far more complicated data handling and analysis controls can be successfully employed for routine operations commonly encountered during mass spectrometric analyses. The application of computers in handling mass spectrometric data opened up many possibilities for accurate and simultaneous measurements of isotopic compositions.

CHAPTER IV THE BACTERIAL REDUCTION OF SULPHATE

4.1 Review

The process of sulphate reduction is widespread in nature. Most sulphides in sedimentary rocks and many elementary sulphur deposits were formed by the bacterial reduction of sulphate. For this reason, geologists, bacteriologists, limnologists, and biogeochemists have begun extensive studies on the formation of mineral deposits by microorganisms. Such research was designed to obtain a better understanding of the kind of bacterial reactions involved in different environments. In particular, the present work strove to evaluate the extent of the isotope effect resulting from the biochemical reduction of sulphur bearing substances in nature.

Sulphate reducing bacteria were first described by BEIJERINCK (1895); the first investigation of the sulphur isotope abundance ratios in sulphides and sulphates, was by THODE, MACNAMARA, and COLLINS (1949). This sulphur isotope study dealt with sulphides and sulphates in sedimentary rock. Soon after, the reduction of sulphate by the bacteria *Desulphovibrio desulphuricans* was observed by THODE, KLEEREKOPER, and MCELECHERAN (1951). This was the first demonstration of the ability of microorganisms to enrich one isotope of sulphur in preference to others during metabolic processes. They

showed that this sulphate reducing bacteria was capable of preferentially selecting the lighter isotope of sulphur, sulphur-32, during the reduction of sulphate to sulphide. Their conclusion was that the sulphides were generally enriched in the lighter isotope, while the sulphates were enriched in the heavier isotope, sulphur-34.

Wallouch, in some unpublished results quoted by THODE, WANLESS, and WALLOUCH (1954), indicated that isotope fractionation during the bacterial reduction of sulphate was temperature dependent. He found that fractionation increased rapidly with a lowering of the temperature. A 1 per cent depletion of sulphur-34 in the hydrogen sulphide at 25°C was observed, reaching 2 per cent at 10°C. This temperature coefficient, however, was much too large to be explained on the basis of a simple kinetic or an equilibrium isotope effect. JONES, STARKEY, FEELY and KULP (1956) showed that with *D. desulphuricans*, the rate of reduction and the concentration of sulphate at all concentrations, were controlling factors in determining the degree of fractionation. These properties of bacterial fractionation of the sulphur isotopes during the reduction of sulphate, were used by THODE (1951, 1954), FEELY and KULP (1957), and JENSEN (1958), in their explanations of a number of phenomena occurring naturally. These works illustrated the usefulness of the isotope tech-

nique in the study of certain geological processes.

KAPLAN, RAFTER, and HULSTON (1960) attempted controlled experiments on various stages of the sulphur cycle. Their results were not encouraging. Numerous processes were found that could cause such fractionation of the isotopes, and many natural valence states of sulphur were recorded. With the wide range of environmental conditions studied, their work served to indicate just how generally applicable such investigations were.

JONES and STARKEY (1957) found the degree of fractionation to be increased at reduced temperatures, as well as at high sulphate concentrations. It was then suggested that temperature regulated the rate of reduction, thereby controlling the enrichment of the sulphur isotopes and the fractionation observed, the degree of fractionation being dépendent on the rate of reduction of the sulphate. JONES and STARKEY (1957) also noted that fractionation was a combination of both chemical and biological reactions. However, it was likely that most natural sulphur formations were biogenic in origin, since temperatures during sulphur deposition were too low for appreciable chemical reduction of sulphate. FEELY and KULP (1957), and HARRISON and THODE (1958), found that the degree of fractionation was inversely proportional to the rate of reduction. That is, greater

isotopic fractionation was observed when the rate of reduction of sulphate was decreased. Similar results were later obtained by KAPLAN, RAFTER, and HULSTON (1950). NAKAI and JENSEN (1960) also reported such observations, however, they used raw cultures. All the previous investigations had used pure cultures of *D. desulphuricans*.

Although attempts were made to interpret these processes in nature in terms of microbiological fractionation of the sulphur isotopes, only HARRISON and THODE (1958) attempted to explain the underlying physiological events. This was partially based on POSTGATE (1952), who established that an intermediate between the sulphate and sulphide was sulphite. Furthermore, the sulphite was observed to be reduced at a faster rate, in the bacterial cell, than the sulphate. HARRISON and THODE (1958) explained this as a reaction mechanism involving two consecutive steps. One step was believed to involve a small isotope effect, with the other producing the large isotope. The first of these steps, the one with the small isotope effect, was suggested to be a result of the uptake of sulphate by the bacteria. The second step was believed to involve the reduction of sulphate to sulphite, the large isotope effect. This large isotope effect would consist of the initial S-O bond breakage occurring in the reduction of sulphate to sulphite.

The observed fractionation was thought to be dependent upon the relative rates of these two steps, that is, the entrance of sulphate into the cell and the breaking of an S-O bond in the reduction to sulphite. HARRISON and THODE (1958) believed these two steps to be competing for control of the rate of reduction. It was of no consequence that the relative rates were closely related to the environment of the bacteria, that is, the temperature, metabolite concentration, and conditions of growth. The question was, which step controlled the reduction rate of the bacteria? Their study concluded that the rate controlling step was the reduction of sulphate to sulphite. This would be expected, since it had been noted by POSTGATE (1952) that sulphite was reduced much more rapidly than sulphate. Any further reduction of sulphite to the end product of a sulphide, could not lead to an isotope effect since the sulphite was reduced as rapidly as it was formed.

KAPLAN and RITTENBERG (1964), unsatisfied with the development of the investigations carried out, re-investigated sulphur isotope fractionation during bacterial sulphate reduction. (They also investigated other important metabolic processes in the sulphur cycle.) Using once more *D. desulphuricans*, KAPLAN and RITTENBERG (1964) studied the effects of temperature and sulphate concentration on the rate of reduction

and the fractionation of the sulphur isotopes. Here the temperature and sulphate concentration, within the normal physiological range of these parameters, influenced the fractionation only in so far as they influenced the reduction rate. Since reduction requires a gain of electrons, they also varied the electron donor. The electron donor previously had been sodium lactate, but now ethanol and molecular hydrogen were also employed. A marked influence on the reduction rate was observed by this variation of the electron donor. Molecular hydrogen was observed to produce the fastest rate of reduction, all other variables remaining constant. Meanwhile, the rate with ethanol was about one-tenth of that observed with molecular hydrogen, whereas lactate was generally about one-half of the hydrogen rate.

With lactate and ethanol as the electron donors, the isotopic fractionation was noted to have been inversely proportional to the rate of reduction. This result was in agreement with HARRISON and THODE's (1958) previous investigation. However, when molecular hydrogen was the electron donor, the fractionation became directly proportional to the rate of reduction of the microorganisms. Strain variations also produced relative changes in the magnitude of the isotopic enrichment, but otherwise no differences were observed due to this modification. It was under such conditions that

the concentration of the metabolite, the temperature, and the electron donor were made to permit similar studies on the reduction of the sulphite ion. The reduction of sulphite was reaffirmed to occur more rapidly than sulphate. The enrichment, however, was usually smaller than during sulphate reduction.

The work of KAPLAN and RITTENBERG (1964) disagreed with the earlier hypothesis of rate controlling steps. Rather, the rate of reduction was always much greater with molecular hydrogen than with either lactate or ethanol as the electron donor. Thus no control of rate occurred during the sulphate reduction, but instead this rate was dependent on the electron donor. It was the availability of electrons at the reduction site, and not penetration of the sulphate, that must have been the rate controlling factor. KAPLAN and RITTENBERG (1964) explained the variety of enrichments found in terms of several possible equilibrium steps prior to a final unidirectional rate controlling step.

KEMP and THODE (1968) undertook a further reexamination of the mechanisms and factors influencing the bacterial reduction of the sulphate ion. Their results hardly differed from those of KAPLAN and RITTENBERG (1964), except that the enrichments and rates with the electron donor ethanol were distinctly different from previous investigations. Although the inverse proportion was still present, the magni-

tude of the reduction was markedly different. One conclusion made was that the passage of H_2S from the cell was fast and hence not a controlling factor in the reduction of sulphate. Their reason for such a suggestion was that the H_2S formed was toxic. That is, the sulphide product was toxic towards the bacterial cell, thus only a small isotope effect was expected in the liberation of the H_2S gaseous product. KEMP and THODE (1968) suggested that the fractionations observed were due to sequential additions of two isotope effects, for both the sulphate and sulphite reductions.

When RAFTER (1967) presented the graphite reduction method, it became logical to pursue the measurement of oxygen isotopes in sulphates. With the oxygen and sulphur isotopic abundance ratios for the sulphate reduction, it would be two tools to "crack the same nut". Hence oxygen isotope effects in sulphates was a complementary process for the understanding of the bacterial reduction of sulphate. LLOYD (1967) reported that in the microbiological reduction of sulphate, the bacteria preferentially metabolized oxygen-16. Hence the δO^{18} value of the residual sulphate in solution became progressively heavier. This was expected, since the oxygen and sulphur isotopes would have most probably followed similar patterns when the bacteria metabolized the sulphate.

A better understanding of sulphate reduction, involving correlations between the sulphur and oxygen isotopic variations, was the aim of the present investigation. The only work done, in addition to the present study, on the relationship between δO^{18} and δS^{34} values of sulphate undergoing bacterial reduction was that of MIZUTANI and RAFTER (1969). Using wet sulphate-free stream mud, MIZUTANI and RAFTER (1969) found the sulphate enriched in oxygen-18 and sulphur-34 relative to the original sulphate used. The ratio of the enrichment of sulphur-34 to the oxygen-18 enrichment in the sulphate, was found to be approximately 4:1. This isotopic enrichment ratio of the sulphate was observed to be independent of the temperature of the reduction. No attempt was made to explain this ratio of the isotopic enrichments.

The bacterial reduction of sulphate has been partially explained in terms of physiological processes rather than physical-chemical reactions. The dependence of isotope fractionation on environmental conditions suggests a wide range of isotopic fractionation factors for many natural processes. Considering its widespread occurrence in nature, it became imperative to better understand these kinds of reactions. For example, the metabolic product of sulphur bacteria plays an important role in organic and inorganic natural phenomena, such as the changes in oxidation-reduction

potentials, the consumption of oxygen, and the precipitation of sulphide minerals. In particular, the present investigation was designed to better evaluate the extent of the isotope effect, and to explain any correlations observed between the sulphur and oxygen isotopic species.

4.2 Microbiological Techniques

During sulphate, sulphite, and elemental sulphur reductions, hydrogen sulphide gas is the end product. In order to facilitate this H_2S evolution, the addition of nutrient, and the medium extractions, a reaction vessel was employed as shown in Figure 4-1. This reaction vessel was a modification of that described by KROUSE, MCCREADY, HUSAIN, and CAMPBELL, (1967). With such a container, nutrient addition or medium extraction could be accomplished without a loss of evolved H_2S gas.

The modified Erlenmeyer flask rested in a thermostatically controlled temperature bath. This constant temperature bath contained distilled water of such a depth as to completely immerse the medium in the reaction flask, yet not interfering with the operation of the sampling port. The reaction flask contained a porous aerator which permitted the flushing gas to immediately disperse in the medium. The product of the bacterial reduction, the H_2S gas, was swept

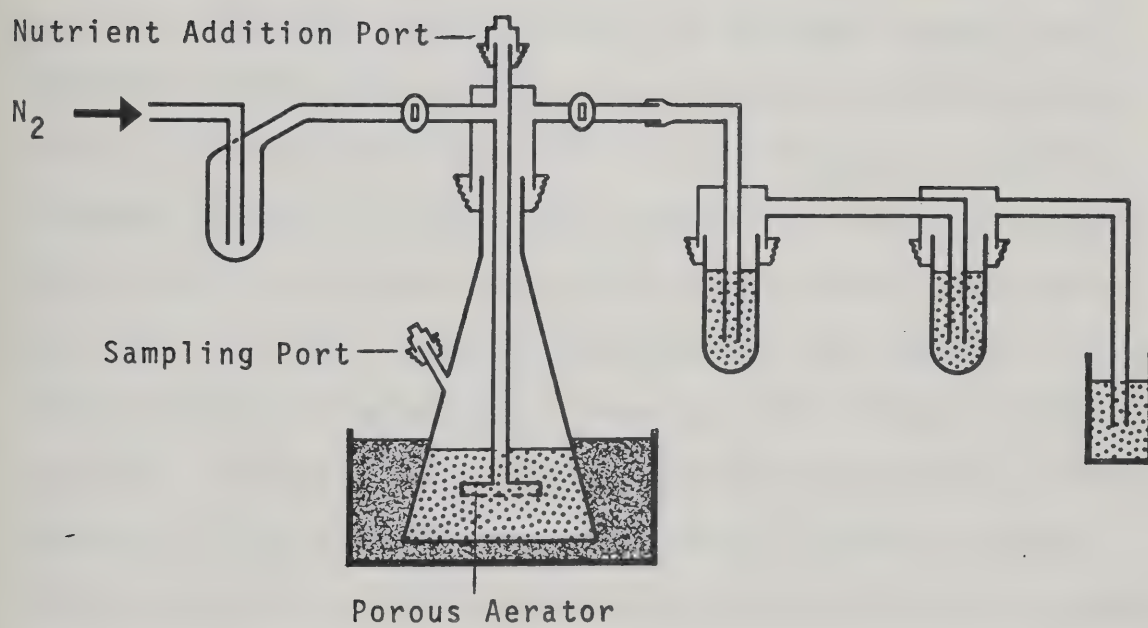


Figure 4-1 Apparatus for the bacterial reduction of sulphate.

continually from the reaction vessel with deoxygenated nitrogen. This was achieved by bubbling nitrogen through a pyrogallol solution, before it entered the reaction vessel, to remove any traces of oxygen not eliminated during the manufacture of the high purity nitrogen gas. (This oxygen scrubbing solution consisted of 10 parts 20 per cent KOH to 4 parts 40 per cent pyrogall, by volume.)

The H_2S , extracted by the nitrogen stream, was bubbled through two scrubbers containing distilled water. It was then trapped from the nitrogen stream by a cadmium acetate mixture, yielding the precipitate cadmium sulphide. The two distilled water scrubbers were employed to remove any HCl vapor from the nitrogen and H_2S gaseous mixture. If the chlorine gas was not removed, then it would have become a contaminant on passage through the acetate solution. Aqueous silver nitrate was added to the acetate solution bearing the yellow CdS precipitate, once the sulphide sample had been removed. The result was a black precipitate Ag_2S . It was subsequently boiled to remove traces of HCl , as well as to induce precipitate self-adhesion. In any one reduction, several H_2S product fractions were collected over chosen time intervals for kinetic isotope analyses. Data was obtained by simply weighing the Ag_2S , quantitatively prepared from the H_2S fractions.

Aseptic conditions were maintained in the reaction vessel by employing two bacteriological filters; one for the entrance of the deoxygenated nitrogen, the other for the nitrogen and sulphide gaseous mixture leaving the reaction flask for the sulphide purification system. The reaction vessel consisted of an Erlenmeyer flask with a sampling port, a nutrient addition port, and a control head, all of which were ground glass joints held tight by small retaining springs. The control head permitted the incoming nitrogen gas to escape through the aerator into the medium, then forced it to exit through the bacterial filter leading to the scrubbing system.

The reaction flask, with all the ground glass port caps secured in place, was autoclaved to produce an aseptic container for the reduction process. The medium after preparation was similarly autoclaved to form the sterile nutrient solution necessary for bacterial growth and subsequent reduction of sulphate without contamination from other sources. For the bacterial reduction of sulphate, the nutrient solution was a modification of "Butlin's" medium (BUTLIN, ADAMS, and THOMAS (1949)). This medium was most convenient since the carbon source for the bacteria also acted as the electron donor during the reduction. This base medium contained:

0.5 g	KH_2PO_4
1.0 g	NH_4Cl
0.1 g	$\text{CaCl}_2 \cdot 6 \text{H}_2\text{O}$
1.0 g	Yeast extract
0.002 g	FeCl_2
3.5 g	60 per cent Na lactate
1000 ml	d. d. H_2O
pH was 7.2	

The original Butlin's medium (BUTLIN, ADAMS, and THOMAS (1949)) contained some sulphates required for good bacterial growth, but these were omitted and FeCl_2 was used so as not to deprive the microorganisms of nutritional requirements, yet at the same time permitting no extraneous sulphate ions to be present in the medium save the known sulphate to be reduced.

The sterile nutrient broth was added aseptically to the autoclaved reaction flask. The inoculum was then added aseptically to the medium in the reaction vessel, and the oxygen free nitrogen gas was then permitted to flush out the air present in the sulphate reduction container. The nitrogen flow rate was adjusted to prevent too vigorous an aeration of the vessel as well as the scrubbing and trapping system. Once the flow was considered satisfactory, the sam-

pling port was opened aseptically and one gram of sterile Na_2SO_4 introduced. The commencement of sulphate reduction usually was observed within about one half an hour after the addition of the sulphate. This was evidenced by the formation of yellow cadmium sulphide precipitate in the sulphide scrubber.

Blank determinations showed that the sulphide contamination from all available sources was negligible. That is, after two days no apparent sulphide was observed in the acetate scrubber. As the reaction proceeded, sulphide samples were removed when considered large enough for isotopic analysis. To determine the rate of sulphate reduction, 90 ml aliquots from the reaction vessel contents were withdrawn at the same time as a sulphide sample was collected. This medium fraction contained sufficient sulphate for a rate determination as well as the measurement of the oxygen and sulphur isotopic composition of the fraction. To avoid further reduction of this sulphate by the microorganisms present in the medium, the 90 ml aliquots were immediately autoclaved after their extraction.

4.3 Chemical Preparation

During the bacterial reduction of sulphate, the modified Butlin's medium provided adequate nutrients for the metabolic processes of the bacteria. One of the by-products

of their metabolism was hydrogen chloride gas. The two scrubbers contained distilled water in which the HCl gas readily dissolved. If the chloride had not been removed before the sulphide was precipitated, it would have dissolved in the acetate solution. Thus when the AgNO_3 was added, the precipitate AgCl would have formed, intimately mixed with the desired Ag_2S product. Its chemical removal could have been done at a later stage of the proceedings, but then it would have become somewhat of a health hazard. The dissolution in water was the fastest and safest method of removing this contaminant. The distilled water in the scrubbers was replaced whenever a sulphate and sulphide fraction were withdrawn.

The acetate solution used to precipitate the H_2S as CdS consisted of a mixture of 500 ml of 17N glacial acetic acid, 62.5 g CdOAc, and 2000 ml of distilled water. The CdS fraction, when removed from the reduction line, was immediately washed by 0.1 N AgNO_3 to form the more stable Ag_2S precipitate. This was then slowly heated to near boiling to induce any chlorine or other undesirable gases to leave the solution, and in addition cause the black precipitate to self-adhere. This usually produced a few conglomerates of sulphide rather than multiple smaller groupings of Ag_2S . As a precaution, NH_4OH was then added, forcing any remaining AgCl precipitate

to become soluble and hence removable. The ammonia vapors made this process quite undesirable, but usually very little, if any, chlorine remained. The resulting Ag_2S precipitate was thoroughly washed at least four times. The fifth washing was used to transfer the sulphide into a 50 ml beaker, then the beaker and its contents were placed in a drying oven at 40°C . After two days the dried Ag_2S precipitate was weighed and then placed in a sealed container until further processing was necessary.

The sulphate and medium fractions extracted from the reaction flask were immediately autoclaved to prevent any further reduction of the sulphate ion. The sterile product was then stored for processing at a later time. Both sulphide and sulphate fractions were withdrawn as the reduction proceeded. Once no further reduction of sulphate was observed, the process was permitted to continue for three days. When no more CdS had formed, the last remanent fraction of sulphide was removed and processed as described. The remainder of the sulphate and medium in the reaction vessel was autoclaved as a precaution, and stored.

The resulting sulphate and sulphide specimens were later chemically treated as a group, thus eliminating any chemical discrepancies between samples of a sulphate reduction.

A few drops of HCl were added to each sulphate plus medium sample to ensure acid conditions, the necessary environment for BaSO_4 but not barium carbonate precipitation. About 40 ml of 0.1N BaCl_2 was added to each sulphate solution to form the desired BaSO_4 precipitate. This fine white precipitate was thoroughly washed three times to rid the solution of remanent medium. A fourth washing was employed to transfer these precipitates into 50 ml beakers. These sulphate specimens were then dried in an oven at 40°C to rid the specimen of water so that accurate weighing could be made of the residual sulphate from the bacterial reductions.

The drying of the sulphate precipitate at low temperatures was found to be the only quantitative technique for extraction of BaSO_4 from solution. Filter paper, centrifuging, and high temperature drying had all been previously attempted without satisfactory results. The fine precipitate could not be quantitatively extracted from the filter paper, nor from the centrifuge tube. High temperature drying tended to give rise to boiling, which in turn caused the precipitate to disperse in the beaker. No such splattering was present when low temperatures were involved. These dried BaSO_4 specimens were then weighed to permit the evaluation of percentage reduction. The resultant BaSO_4 samples were then divided into two portions; the

larger portion for oxygen isotopic abundance ratio measurements of the sulphate, and the smaller portion for the determination of the sulphur isotopic composition of the sulphate.

The BaSO_4 designated for oxygen isotopic abundance ratio measurements was already in a suitable form for the direct reduction by graphite. These samples were reduced by the carbon reduction method already described. The resultant CO_2 product was then analyzed mass spectrometrically for the oxygen isotopic composition of the unreacted sulphate from the bacterial reductions.

The BaSO_4 designated for sulphur isotope studies was not in a convenient form. It was necessary to chemically reduce the sulphate to sulphur dioxide, through a series of chemical reactions (RAFTER (1957), GAVELIN, PARWELL, and RYHAGE (1960), THODE, MACNAMARA, and DUNFORD (1961), and RICKE (1964)). The method of THODE, MACNAMARA, and DUNFORD (1961) was the technique basically adopted for the present study. The BaSO_4 designated for sulphur isotope studies was placed in a 200 ml flask as shown in Figure 4-2. This reduction flask was fitted by means of a ground glass joint and retaining springs to the lower end of a reflux container. The addition of a reducing agent to the BaSO_4 sample resulted in H_2S evolution. This sulphide gas was forced by a nitrogen

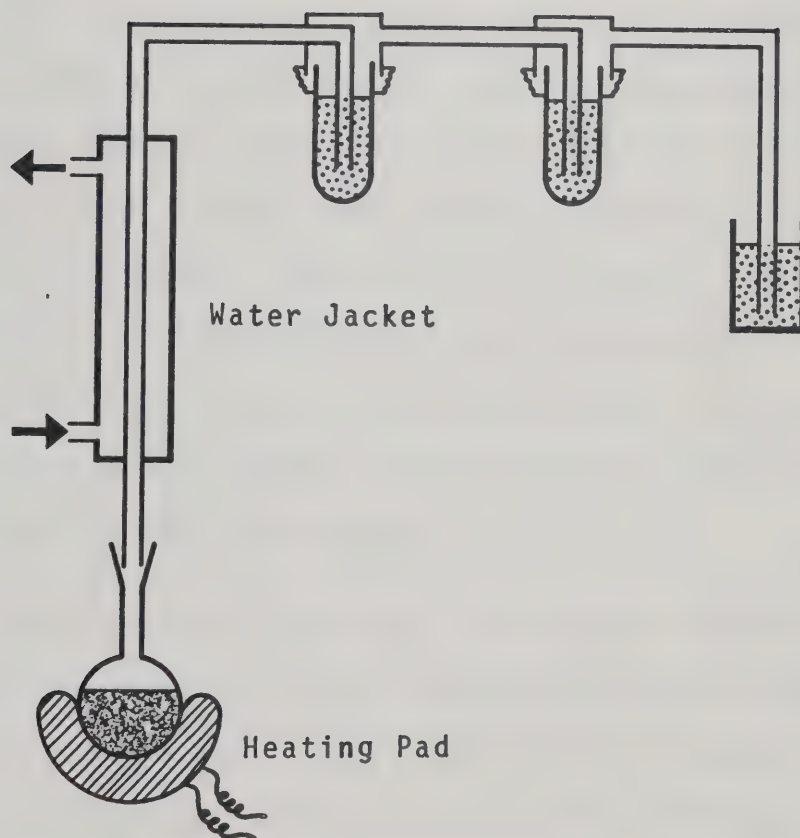


Figure 4-2 Apparatus for the chemical reduction of sulphate.

stream through the water jacket to the distilled water and acetate scrubbers. The distilled water was employed to remove any chlorine gas evolved from the reduction process due to the breakdown of the reducing reagent. The H_2S was trapped as CdS as outlined previously. This chemical reduction of sulphate proceeded in a similar manner to the bacterial reductions except much faster, since there were heating jackets placed around the flasks containing the sulphate and reduction mixture. A reflux condenser was necessary to prevent the reduction mixture from evaporating. This process involved a negligible isotope effect because there was nearly 100% conversion.

The chemical reduction of sulphate was brought about by means of a very strong reduction mixture consisting of 500 ml HI, with 816 ml of concentrated HCl, and 245 ml of 50 per cent H_3PO_2 . This was produced by careful addition of these acids, then the resultant mixture was boiled for 45 minutes to expel any H_2S or chlorine gases. Heating jackets were employed to maintain moderate boiling of the reduction mixture. The 30 mg sulphate sample usually required about one

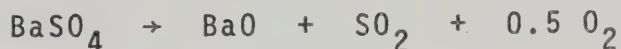
hour for its quantitative chemical reduction to sulphide.

The resultant CdS product was precipitated as Ag_2S by the addition of 0.1N AgNO_3 , boiled, dried, and weighed as previously described in section 4.2. This Ag_2S was then converted to SO_2 so that the sulphur isotopic composition of the unreacted sulphate could be determined. (This process was also used to convert the sulphide product of the bacterial reductions to SO_2 for sulphur isotopic abundance ratio measurements.) The Ag_2S was placed in a quartz boat and burned in O_2 to form SO_2 , which could be readily analyzed mass spectrometrically for the sulphur isotopic content. The SO_2 product of the Ag_2S burning was formed following the procedures of RAFTER (1957), HULSTON and SHILTON (1958), and THODE, MONSTER, and DUNFORD (1961). Here the Ag_2S was placed in a quartz boat in a furnace at 1200°C , and purified oxygen gas then passed over the hot silver sulphide. The SO_2 so formed was quantitatively measured after the removal of any CO_2 , water vapor, or O_2 contaminants.

Unfortunately this conversion of BaSO_4 to SO_2 , for the determination of sulphur isotopic abundance ratios in sulphate, involved multiple operations. There was the chemical reduction liberating H_2S , then the tedious double

precipitation, and finally the oxygen burning which liberated the desired SO_2 . Each procedure caused no appreciable fractionation of the isotopes, but involved very tedious and time consuming operations. The chief disadvantage of any such technique to convert BaSO_4 to SO_2 was the requisite time and labor. Such procedures did consume a major part of the experimental effort in the present research program. Recently, HOLT and ENGELKEMEIR (1970) described a method by which BaSO_4 was rapidly converted to SO_2 in one operation. This method is to be recommended for any study where BaSO_4 must be converted to SO_2 .

HOLT and ENGELKEMEIR (1970) investigated the thermal decomposition of BaSO_4 . The sulphate was covered with pulverized quartz powder in a fused quartz tube and heated in vacuum to the softening point of quartz. At such a temperature, about 1400°C , the reaction



yielded SO_2 , which was collected in a cold trap. The O_2 was then pumped away while the BaO fused with the silica surroundings. This method, however, did not assure a uniform oxygen-18 abundance in the SO_2 as did the procedures involving sulphide samples oxidized by oxygen from a common source. A correction

for such an oxygen-18 interference was made from measurements of both the 66/64 and the 50/48 mass ratios.

The yields from such thermal decompositions of BaSO_4 were always greater than 98 per cent of the theoretical yield. The chief impurity found was CO_2 . The gas produced usually consisted of about 99 per cent SO_2 , 0.5 per cent CO_2 , and 0.5 per cent other impurities. The other impurities were mainly water and mass 28 materials, that is, N_2 and CO . No SO_3 was detected and only traces of CS_2 and COS were observed. Aside from the obvious advantages of time and labor, samples from about 5 mg to 50 mg were quantitatively converted.

4.4 The Bacterial Reductions

A series of bacterial sulphate reduction experiments were conducted at 24°C and 30°C , using 1000 mls of the modified Butlin's medium as the culture media and 1 g of Na_2SO_4 . Oxygen free nitrogen was swept through the reaction vessels as previously described. The inoculum however, was not just one strain of a sulphate reducing bacteria, such as *D. desulphuricans* employed in previous investigations.

To help elucidate the mechanisms of sulphate reduction to sulphite as well as the further reduction of sulphite to sulphide, the inoculum consisted of two strains of bacteria. One strain, *Bacillus 8P*, was a sulphate reducer

unable to metabolize sulphite; whereas the other strain, *Clostridium Dm 3*, was a sulphite reducing bacteria which would yield the final H_2S product, but was unable to utilize sulphate. Hence the two bacterial strains had distinctive functions. The final H_2S product was only the result of a double reduction. Unreacted sulphate indicated the *Bacillus 8P's* inability to further reduce sulphate, while an accumulation of intermediate sulphite suggested the *Clostridium Dm 3's* inability to reduce any more sulphite. By observing the amount of sulphide, unreacted sulphate, and sulphite intermediate at various stages of the reduction, an attempt could then be made to more clearly understand the mechanisms involved in the bacterial reduction of the sulphate ion.

The sterile medium in the aseptic reaction vessel was inoculated with these two bacterial strains. As the microbiological reduction proceeded, the deoxygenated nitrogen forced the H_2S product through the sulphide purification system. Fractions of the sulphide and medium (containing the unreacted sulphate and sulphite) were withdrawn throughout the reduction process. These products were treated as previously described. This two strain inoculum was used for the reduction of sulphate on four separate occasions. Two reductions were carried out at $24^{\circ}C$ and two more at $30^{\circ}C$, to ascertain temperature dependency of the reduction rate.

The amounts of media, inoculate, and sulphate concentrations were identical in all four cases.

Of the twelve bacterial reduction-of-sulphate experiments investigated during the course of the present study, these four were singled out for a most intense investigation. Most of the other sulphate reductions were long term experiments employing only one bacterial strain for the reduction of sulphate to sulphide. These reductions were considered unsatisfactory at the time because of the apparent lack of completeness of reduction. That is, the reduction of sulphate was usually less than 30 per cent of that believed possible, although most adequate precautions had been taken to ensure that the reducing bacteria had all the nutrients required for a complete reduction of the available sulphate. (No real understanding of the sulphate reduction was believed possible with such poor yields.) The isotopic composition of the sulphate fractions were determined for some of these runs. No appreciable variations in the isotopic abundance ratios were observed as the reaction proceeded. Even when the bacterial reduction of sulphate had completely ceased, the isotopic enrichments were not as substantial as expected.

The concept of two distinctively different strains, with different metabolisms, was the second simplest form of sulphate reduction by microorganisms. With no apparent success with only one strain, the employment of two strains was the most obvious choice. To eliminate any confusion arising from the double reduction, it was decided to use strains which had no common sulphur metabolic processes. With the synergism of the $\text{SO}_4\text{-SO}_3^*$ reducer unable to reduce sulphite and the $\text{SO}_3\text{-H}_2\text{S}^*$ reducing bacteria incapable of reducing sulphate, the intermediate sulphite observed by POSTGATE (1952) could be independently evaluated.

KAPLAN and RITTENBERG (1964) and KEMP and THODE (1968) concluded that the rate of reduction was inversely proportional to the fractionation of the sulphur isotopes if lactate or ethanol was the electron donor. With such a donor, it became apparent that the rate of reduction should be slow, but how slow became a very important question. A total reduction time of about a week was considered much too short, yet months could have resulted in the possibility of no significant results, as the previous sulphate reductions had shown. The optimum time was considered to be in the order of about six weeks. Then arose the problem of finding both a $\text{SO}_4\text{-SO}_3$ and a $\text{SO}_3\text{-H}_2\text{S}$ reducer which were compatible, totally dissimilar in metabolic functions involving sulphur-

(* $\text{SO}_4^=$ and $\text{SO}_3^=$ for convenience in the remainder of the text are simply SO_4 and SO_3 ; charge omitted. However, SO_2 and H_2S refer to the gaseous forms.)

bearing ions, yet such that the reduction process would be complete in about six weeks.

The selection and isolation of such a pair of microorganisms was no simple task. Dr. F. Cook personally undertook this tedious task and provided the two strains employed (unpublished work by Dr. F. D. Cook).

During the reduction experiments, fractions of sulphate and sulphide were withdrawn, as recorded in Table 4-1. The total amount of sulphur used was calculated from the amount of Na_2SO_4 added. With a knowledge of H_2S evolved and sulphate removed, the amount of intermediate was determined. (This intermediate was logically the product of the sulphate reduction, and the known material needed by the sulphite reducing bacteria.) The accumulation of this intermediate was evaluated as shown in Figure 4-3. This was calculated from a sulphur mass balance. Also, knowing the concentration of the unreacted sulphate, the medium volume, and the sulphide evolved, the instantaneous sulphur mass balance for a particular sample (n) can be evaluated using

$$((\text{mg S})_{\text{BaSO}_4} + (\text{mg S})_{\text{Ag}_2\text{S}} + (\text{mg S})_{\text{int}})_n = ((\text{mg S})_{\text{BaSO}_4} + (\text{mg S})_{\text{int}})$$

A sulphur balance was required to obtain the concentration of the sulphite ions, because any oxygen mass balance would have involved oxygen from the medium constituents. This oxygen

bacterial reductions of sulphate

Specimen Number	Time (days)	BaSO ₄ (mg)	Ag ₂ S (mg)	S in BaSO ₄ (mg/l)	S in Ag ₂ S (mg/l)	Calc. S int. (mg/l)
1 - 0	0	--	--	226	--	--
1 - 1	6	95	130	145	17	64
1 - 2	8	92	65	140	9	60
1 - 3	11	90	109	137	17	46
1 - 4	19	81	83	124	15	44
1 - 5	41	66	40	100	8	60
2 - 0	0	--	--	226	--	--
2 - 1	6	121	131	185	17	24
2 - 2	8	118	85	180	12	17
2 - 3	11	111	119	170	19	8
2 - 4	19	109	61	166	11	1
3 - 0	0	--	--	226	--	--
3 - 1	6	82	97	125	13	88
3 - 2	8	83	87	127	12	74
3 - 3	11	80	80	122	13	66
3 - 4	19	81	68	124	12	52
4 - 0	0	--	--	226	--	--
4 - 1	6	106	90	162	12	52
4 - 2	8	102	79	156	11	47
4 - 3	11	105	68	160	11	32
4 - 4	19	98	69	150	12	30
4 - 5	41	n. d.	28	n. d.	6	n. d.

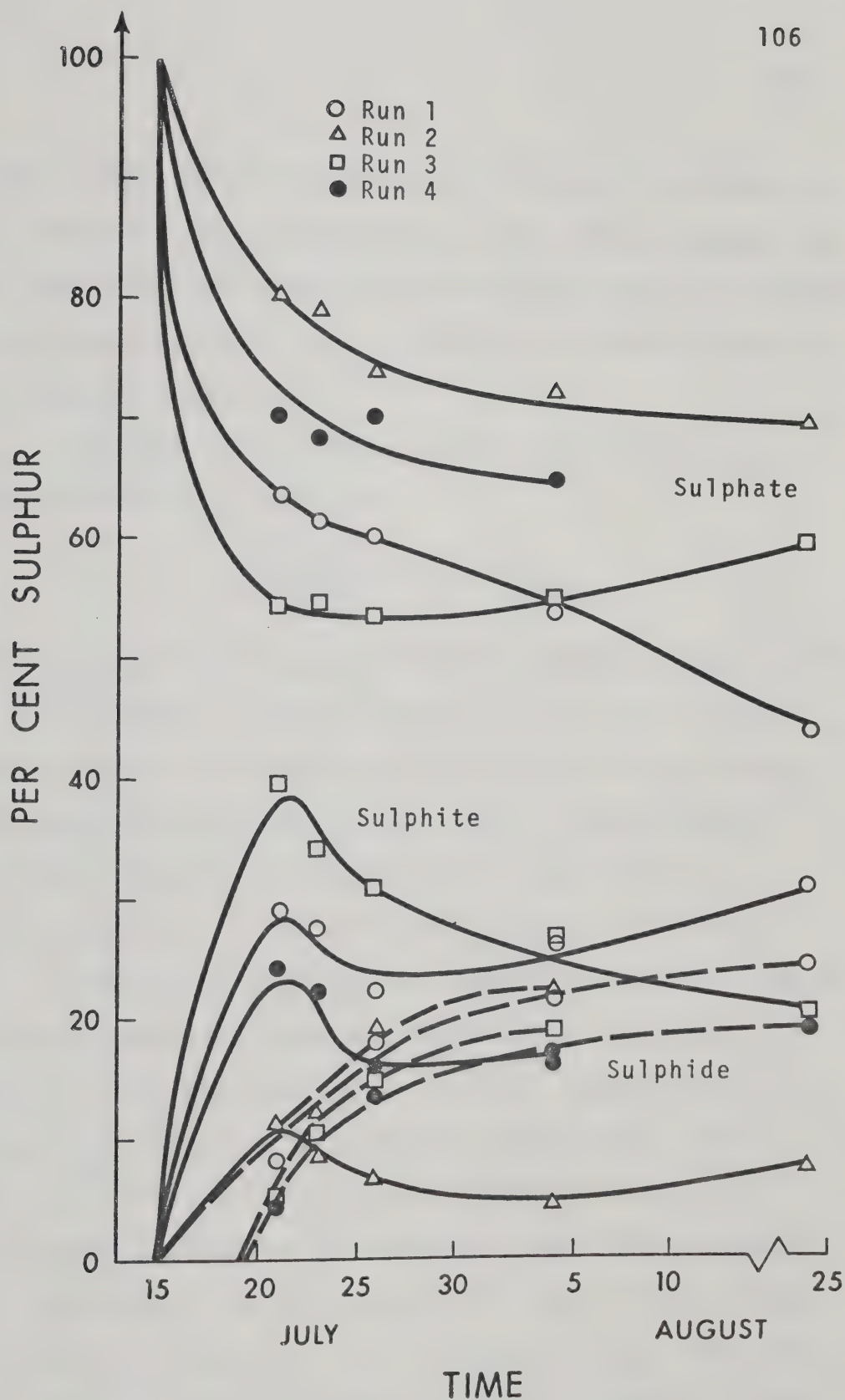


Figure 4-3 Sulphur distribution during the bacterial reduction of sulphate.

was from so many possible sources that isotopic analyses of the CO_2 evolution were meaningless. Most of the oxygen did in fact come from the reduction of sulphate, but the proportion from extraneous materials was sufficient to render fruitful interpretations impossible.

The mass spectrometric results obtained were tabulated using the δ notation:

$$\delta = \left(\frac{R_{\text{sample}}}{R_{\text{std}}} - 1 \right) \times 1000 \quad ,$$

where R was the $\text{S}^{34}/\text{S}^{32}$ or the $\text{O}^{18}/\text{O}^{16}$ abundance ratio of the sample and standard. The enrichment of both the sulphur and oxygen species in the unreacted sulphate at any stage of the reduction was noted in Table 4-2. The unreacted sulphate was progressively enriched in oxygen-18 and in sulphur-34 relative to the original sulphate introduced.

To avoid calibration of isotopic standards, one of the sulphate reduction samples, the original unreacted sulphate 1-0, was employed as an internal standard for all four bacterial reduction of sulphate experiments. The graphite used for the reduction of sulphate to CO_2 , for oxygen isotope studies of the sulphate, was from a common source. Similarly the oxygen used for the burning of the Ag_2S to yield SO_2 was also from a common source. Thus the internal standard used for both oxygen and sulphur isotopic

Table 4-2 Oxygen and sulphur isotopic variations
during the bacterial reduction of sulphate

Sample Number	Per Cent Reaction	Sulphate (‰)		Sulphide (‰)
		δS^{34} wrt	δO^{18} wrt	δS^{34} wrt
		1 - 0	1 - 0	1 - 0
1 - 0	0	0	0	--
1 - 1	35.8	+2.16	+0.58	-13.74
1 - 2	38.1	+2.02	+0.47	-25.70
1 - 3	39.4	+1.98	+0.44	-25.21
1 - 4	45.1	+2.65	+0.66	-24.69
1 - 5	55.8	+3.73	+0.90	-49.27
2 - 0	0	0	0	--
2 - 1	18.1	+1.93	+0.46	-14.15
2 - 2	20.3	+1.33	+0.32	-28.67
2 - 3	24.8	+2.45	+0.69	- 0.38
2 - 4	26.5	+3.84	+1.02	n. d.
3 - 0	0	0	0	--
3 - 1	44.7	+1.11	+0.26	+ 0.66
3 - 2	43.8	+2.16	n. d.	- 6.78
3 - 3	46.0	+1.37	+0.33	- 4.89
3 - 4	45.1	+3.34	+0.86	- 8.99
4 - 0	0	0	0	--
4 - 1	28.3	+1.35	+0.33	- 3.48
4 - 2	31.0	+1.94	+0.49	-14.06
4 - 3	29.2	+1.15	+0.29	-21.25
4 - 4	33.6	+1.58	+0.45	-27.23
4 - 5	n. d.	n. d.	n. d.	-24.77

$$\text{Per Cent Reaction} = \left(1 - \frac{\text{conc. of sulphate}}{\text{initial conc.}} \right) \cdot 100 \%$$

standards involved no carbon or oxygen corrections.

The apparent non-linearity of the isotopic data took on a new dimension when the per cent H_2S production was considered. Figure 4-4 yielded most intriguing sulphur and oxygen isotopic abundance ratios if the general trend of each reduction was considered. The depletion or enrichment of these isotopic species was totally sympathetic. These variations in isotopic composition became more startling when the relationship between the $\text{O}^{18}/\text{O}^{16}$ and $\text{S}^{34}/\text{S}^{32}$ ratios were plotted as figure 4-5. Here the relative enrichments of sulphur-34 to oxygen-18 yielded a ratio of about 4:1. This ratio in the sulphate was observed to have been independent of the temperature of the reduction.

Variations in the isotopic composition of the unreacted sulphate were most probably a result of the metabolic activity of the sulphate reducing bacteria constituting part of the inoculum. However, the remainder of the inoculum, the sulphite reducing bacteria, were capable of producing variations in the isotopic composition of the sulphate. The effects of their metabolism were believed recorded only in the sulphur isotopic composition of the H_2S gas evolved. The isotopic data from the final sulphide product was noted in Table 4-2 and presented as Figure 4-6.

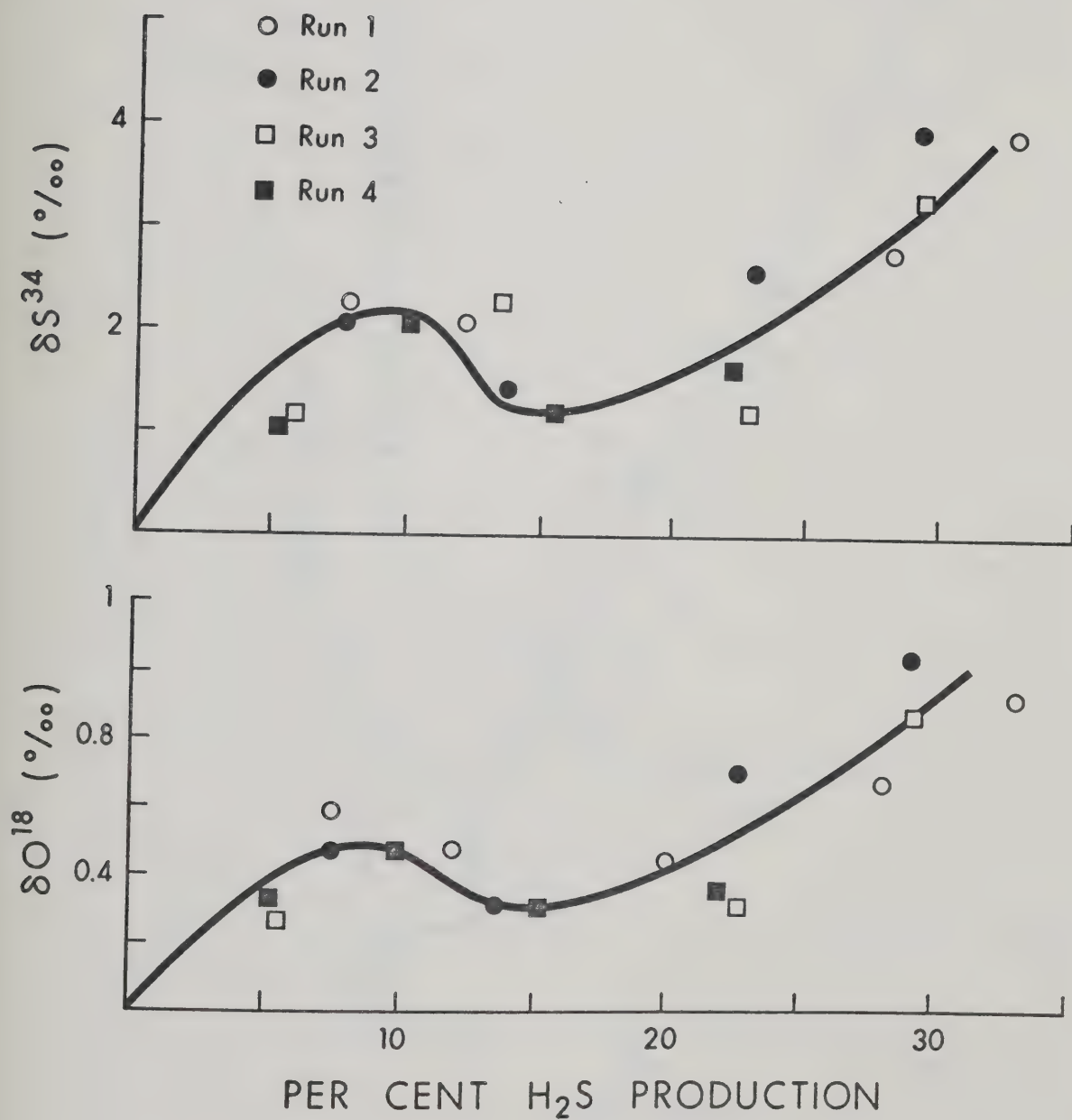


Figure 4-4 δS^{34} and δO^{18} values for unreacted sulphate as a function of the H_2S production.

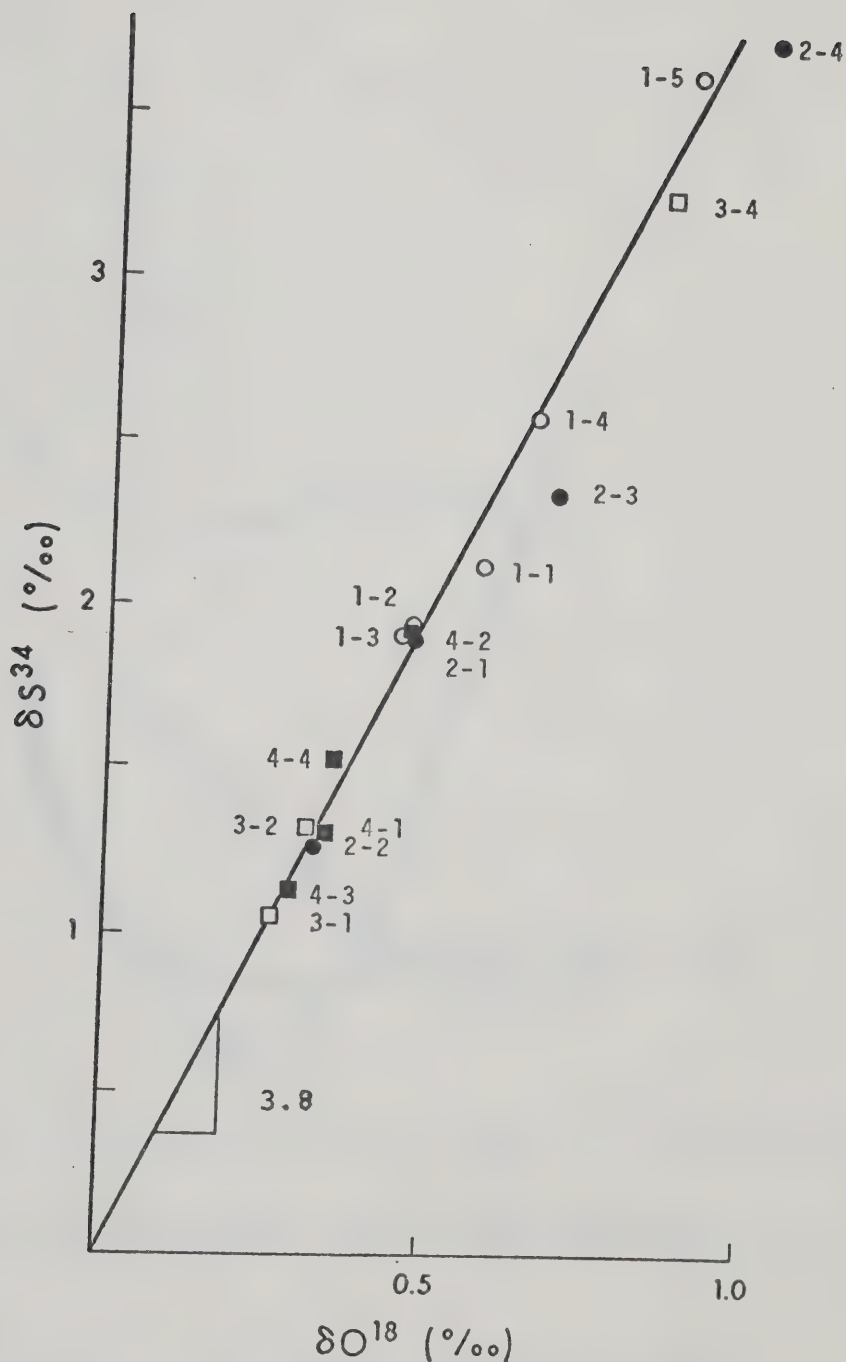


Figure 4-5 Relationship between δO^{18} and δS^{34} values of sulphate during bacterial sulphate reduction.

N. B. Calculation of the best fitting straight line yields a slope of 3.82 ± 0.22 . Errors of 0.05 ‰ in both coordinates yield a sum of square residuals of 7.3 with 14 degrees of freedom indicating the individual measurement precision probably better than 0.05 ‰ .

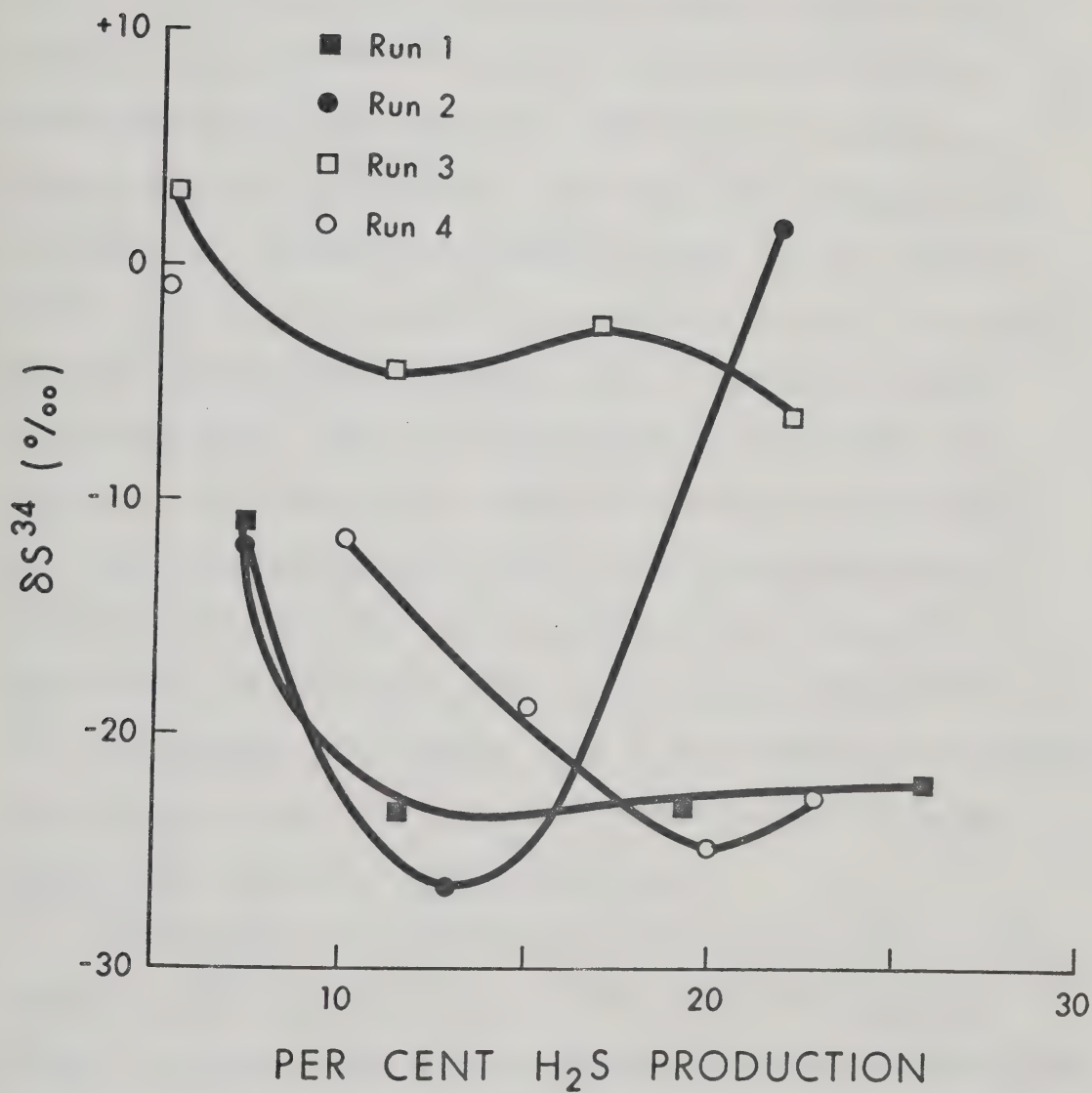


Figure 4-6 δS^{34} values of the H₂S evolved versus the per cent H₂S production.

4.5 Discussion

Since the development of oxygen isotopic abundance ratio measurements in sulphate, no investigators have studied the sulphur and oxygen isotopic correlations observed during the bacterial reduction of sulphate, except MIZUTANI and RAFTER (1969) and the present study. In addition, MIZUTANI and RAFTER (1969) did not conduct their investigation with known microorganisms, but rather with an indeterminate conglomerate of organisms found in stream mud. Hence microbiological conclusions were limited. An analysis of sulphate reduction mechanisms was also impossible with such a complicated medium, in spite of the fact that such conditions are naturally occurring. The understanding of biological metabolisms in such complex environments, and in particular the reduction of sulphate, requires intensive investigation if such mechanisms are to be fully understood.

Differences in temperature of the reductions in the present study were observed to alter the fractionation of the isotopes. The first sulphate fraction removed from the reaction flask (Figure 4-4) showed most clearly that the fractionation was greater for reductions 1 and 2 at 24°C , than for runs 3 and 4 at 30°C . (This information was basically masked by the inversions, but witnessed most clearly in runs 1 and 4 with the sulphur isotopic composition of the unreacted sulphate.) This confirmed the observations of earlier investigators. Here a decrease in temperature

caused a decrease in the rate of reduction, which in turn caused an increase in the fractionation. Temperature was only considered important in so far as it modified the rate of reduction.

The metabolic activity of the sulphate reducer became apparent when observing the unreacted sulphate. The relative enrichment ratio was expected, but not the isotopic inversions recorded in both the sulphur and oxygen isotopic data. These variations were thought to have been transferred to the sulphite produced. The sulphite reduction was monitored by observation of the H_2S product (Figure 4-6 shows the sulphur isotopic composition of the sulphite product.) Inversions did appear, but no apparent correlation between the two reduction processes was at first obvious.

A comparison of the relative fractionation of the sulphite and sulphate was thought to be an aid in determining the mechanisms of $\text{SO}_4 \rightarrow \text{SO}_3 \rightarrow \text{H}_2\text{S}$ reduction. A plot of the sulphur isotopic variations in the sulphide and unreacted sulphate is shown in Figure 4-7. The same sulphide to sulphate comparison is presented in Figure 4-8 using MIZUTANI and RAFTER's (1969) data. Similar radically varying relative fractionations were observed.

In both MIZUTANI and RAFTER's (1969) study and the present investigation, the slope at any instant was a comparison of the fractionation of sulphur isotopes in the sulphide,

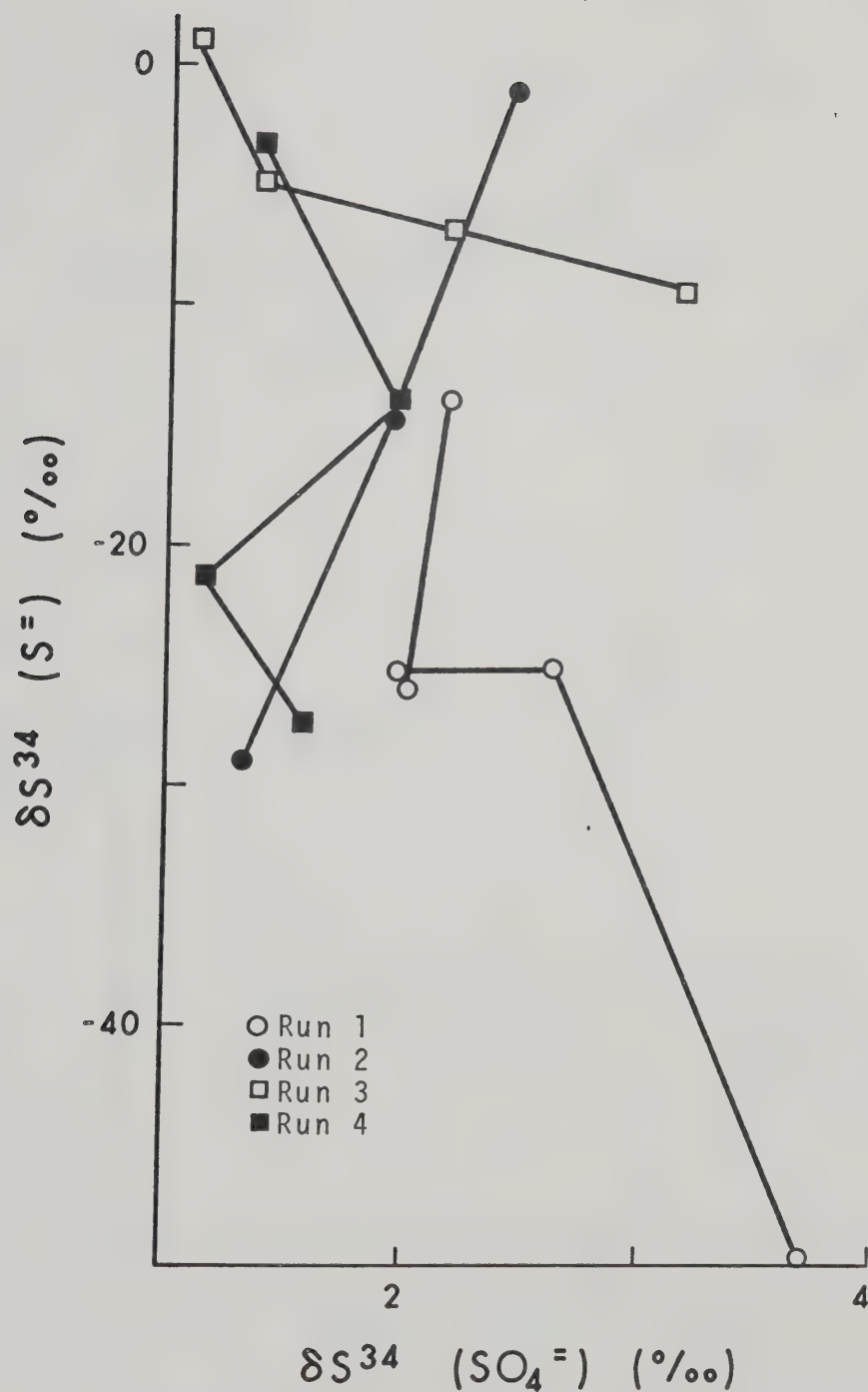


Figure 4-7

δS^{34} of the sulphide versus the δS^{34} values in sulphate (present study).

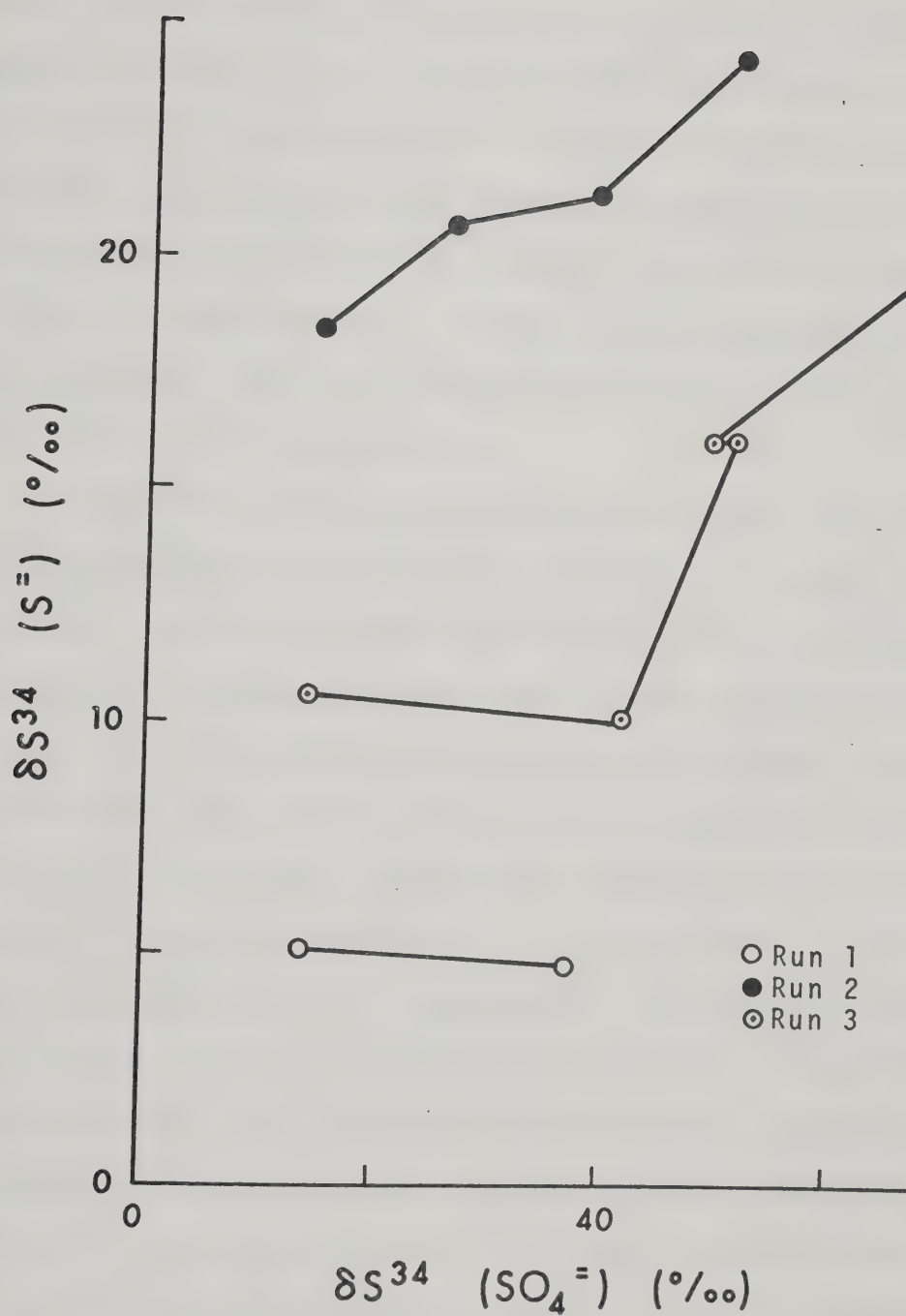


Figure 4-8 δS^{34} of the sulphide versus the δS^{34} values in sulphate (Mizutani and Rafter (1969)).

to the fractionation of the sulphur isotopes in the unreacted sulphate. The slope variations implied that the sulphite fractionation and the sulphate fractionation were not always consistent. (The isotopic inversions of the present investigation did not appear to cause any change in the rate proportionality. Runs 2 and 3 compared favorably to reductions 1 and 2 of MIZUTANI and RAFTER (1969), where no inversions were observed.)

It has been established (Section 4.1) that the sulphate fractionation rate is inversely proportional to the rate of reduction. However, since both studies under consideration have recorded instances where the sulphite fractionation rate was both inversely and directly proportional to the sulphate rate of fractionation, it was concluded that both inversely and directly proportional reduction rates were present during the same reduction process (runs 1 and 4 of figure 4-7, and run 3 of figure 4-8). Hence sulphite was reduced both faster and slower than sulphate. Thus the rate controlling step was sulphate and/or sulphite reduction, depending upon which of the 7 reductions was under consideration. It was hard to conceive of such oscillations in the rate control during a single bacterial reduction experiment. Either both sulphate and sulphite reductions were rate controlling and each competing for control of the rate, or else some other factor controlled the rate of the $\text{SO}_4 \rightarrow \text{SO}_3 \rightarrow \text{H}_2\text{S}$

reduction. The complexity of this rate control did seem to become more obvious as the temperature of the reduction increased.

One advantage of employing two strains of bacteria which performed distinctly different metabolic processes, is that the intermediate was known to have been sulphite. The size of this reservoir would definitely elucidate the predominant rate controlling step during a particular sulphate reduction. If the $\text{SO}_4 \rightarrow \text{SO}_3$ reduction was rate controlling, then there would be no sulphite ions present in the reaction flask once the reduction had ceased. On the other hand, if the $\text{SO}_3 \rightarrow \text{H}_2\text{S}$ reduction was rate controlling, then when the reduction ceased, sulphite would be in solution since it had not been completely reduced by the sulphite reducing bacteria. Thus the double reduction permitted both an isotopic and mass balance, which would yield the amount of the intermediate sulphite present during the reduction, as well as the isotopic composition of this intermediate. These calculations, however, could not be performed on the oxygen data since no reliable oxygen bearing product was liberated, hence only the sulphur isotopes were used for the isotopic and mass balances. At any instant during the bacterial reduction of sulphate, the mass balance yielded

$$100\% = (\% \text{ mg S})_{\text{H}_2\text{S}} + (\% \text{ mg S})_{\text{un SO}_4} + (\% \text{ mg S})_{\text{int.}},$$

where the amount of sulphur in that available system must equal the amount removed as sulphide, plus the amount remaining as unreacted sulphate, plus the amount accumulated as intermediate. The instantaneous isotopic balance yielded

$$(\delta \text{S}^{34})_{\text{total S present}} (100\%) = (\delta \text{S}^{34})_{\text{H}_2\text{S}} (\% \text{ H}_2\text{S}) + (\delta \text{S})_{\text{un SO}_4} \cdot (\% \text{ un SO}_4) + (\delta \text{S})_{\text{int SO}_3} (\% \text{ int SO}_3)$$

Here the isotopic composition ($^0/_{\infty}$) of the total sulphur present must be equal to the per cent isotopic compositions of the sulphide product, the unreacted sulphate, and the sulphite intermediate. Once the amount of sulphur tied up as intermediate was determined from the instantaneous mass balance, then its instantaneous isotopic composition was evaluated from the isotope balance. These instantaneous values are recorded in Table 4-3, and reflect the behavior of the system. (These calculations were only applicable for

Table 4-3 Instantaneous mass and isotopic balances calculated to determine the behavior of the sulphite intermediate

Specimen Number	Inst. Mass Balance (% of S available)			Inst. Isotopic Balance (δS^{34} , ‰)		
	<u>H₂S</u>	<u>un SO₄</u>	<u>int SO₃</u>	<u>H₂S</u>	<u>un SO₄</u>	<u>int SO₃</u>
1 - 1	7.5	64.2	28.3	-13.74	+2.16	-1.26
1 - 2	4.3	67.0	28.7	-25.70	+2.02	+3.00
1 - 3	8.5	68.5	23.0	-25.21	+1.98	+13.46
1 - 4	8.2	67.8	24.0	-24.69	+2.65	+21.49
1 - 5	4.8	59.5	35.7	-49.27	+3.73	+21.61
2 - 1	7.5	81.9	10.6	-14.15	+1.93	-4.90
2 - 2	5.8	86.1	8.1	-28.67	+1.33	-7.81
2 - 3	9.6	86.3	4.1	-0.38	+2.45	-37.51
2 - 4	6.2	93.3	0.5	n. d.	+3.84	n. d.
3 - 1	5.8	55.3	38.9	+0.66	+1.11	-1.68
3 - 2	5.6	59.6	34.8	-6.78	+2.16	-2.73
3 - 3	6.5	60.7	32.8	-4.89	+1.37	-0.48
3 - 4	6.4	66.0	27.6	-8.99	+3.34	-3.29
4 - 1	5.3	71.7	23.0	-3.48	+0.33	-0.23
4 - 2	5.1	72.9	22.0	-14.06	+0.49	+2.50
4 - 3	5.4	78.8	15.8	-21.25	+0.29	+11.89
4 - 4	6.3	78.1	15.6	-27.23	+0.45	+23.32
4 - 5	3.3	n. d.	n. d.	-24.77	n. d.	n. d.

(The δS^{34} value of the initial sulphate was taken as 0 ‰ ; the amount of sulphur in the initial sulphate was = 226 mg, and for these experiments 90 ml aliquots were withdrawn.)

times when the sulphate and sulphide fractions were withdrawn.) The isotopic composition of the intermediate sulphite was not always observed to lie between that of the unreacted sulphate and the H_2S product, as shown in Figures 4-9, 4-10, 4-11, and 4-12.

Isotope fractionation during the $\text{SO}_4 \rightarrow \text{H}_2\text{S}$ reduction was possible, since there was a large sulphate reservoir available for sulphate reduction to sulphite. The $\text{SO}_3 \rightarrow \text{H}_2\text{S}$ reduction however, had a smaller reservoir. Even with such a limited reservoir, the H_2S should have been isotopically lighter than the sulphite intermediate. As long as sulphite was permitted to accumulate, the sulphide usually has been lighter than the corresponding sulphite. When the isotopic composition of the intermediate remained between the sulphate and sulphide isotopic abundance ratios, it was assumed that there was sufficient sulphite present to have permitted isotope selection by the sulphite reducing bacteria. Such a situation seems to have occurred in the third reduction (Figure 4-11). The sulphate reduction rate was faster than that of the sulphite reduction, hence a sulphite reservoir was present. As the sulphite reservoir increased in size, the $\text{SO}_3 \rightarrow \text{H}_2\text{S}$ reduction preferentially metabolized the lighter sulphur-32. The H_2S product was observed to become isotopically lighter. From Figure 4-11 it appears

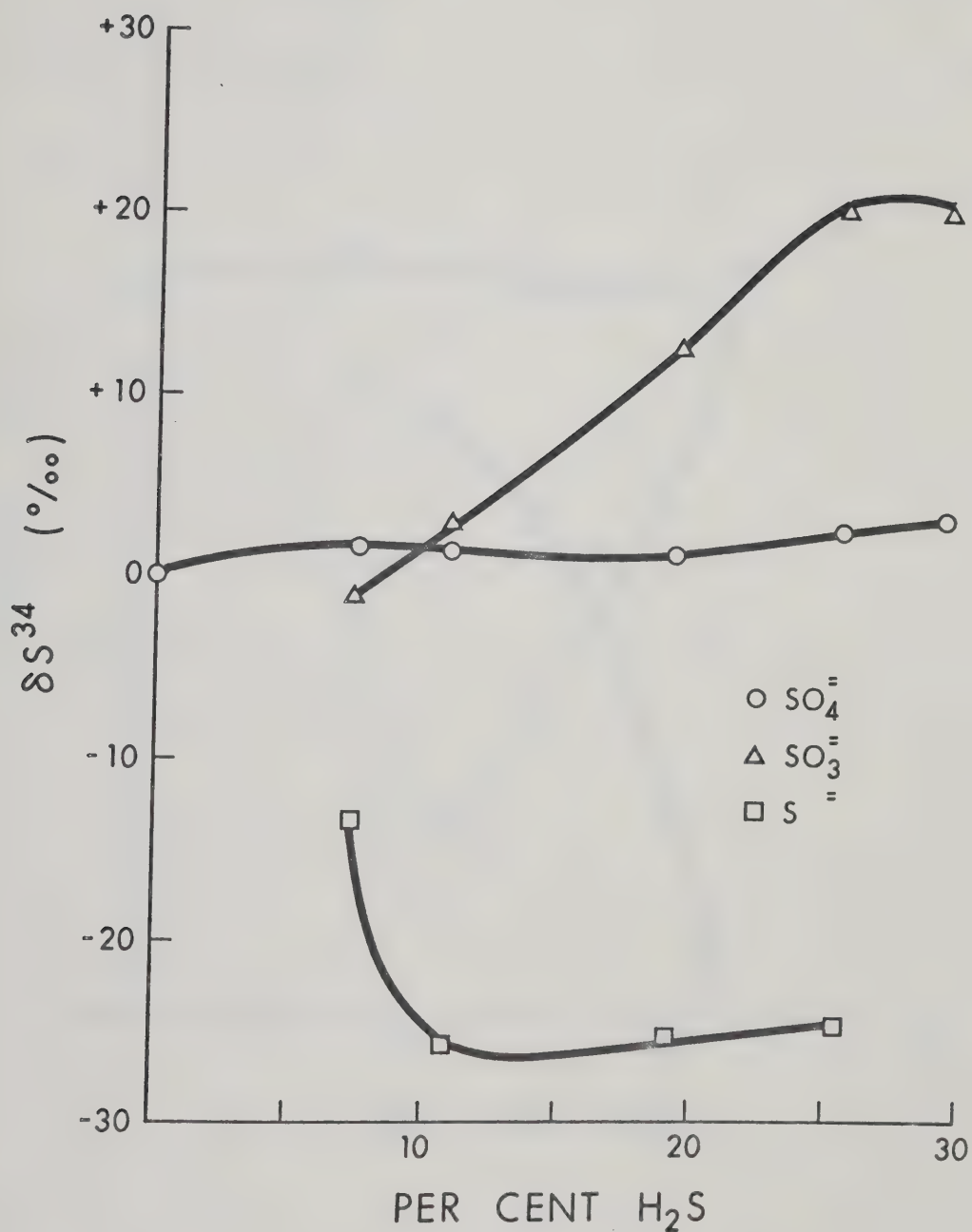


Figure 4-9 Isotope effects during bacterial reduction No. 1 .

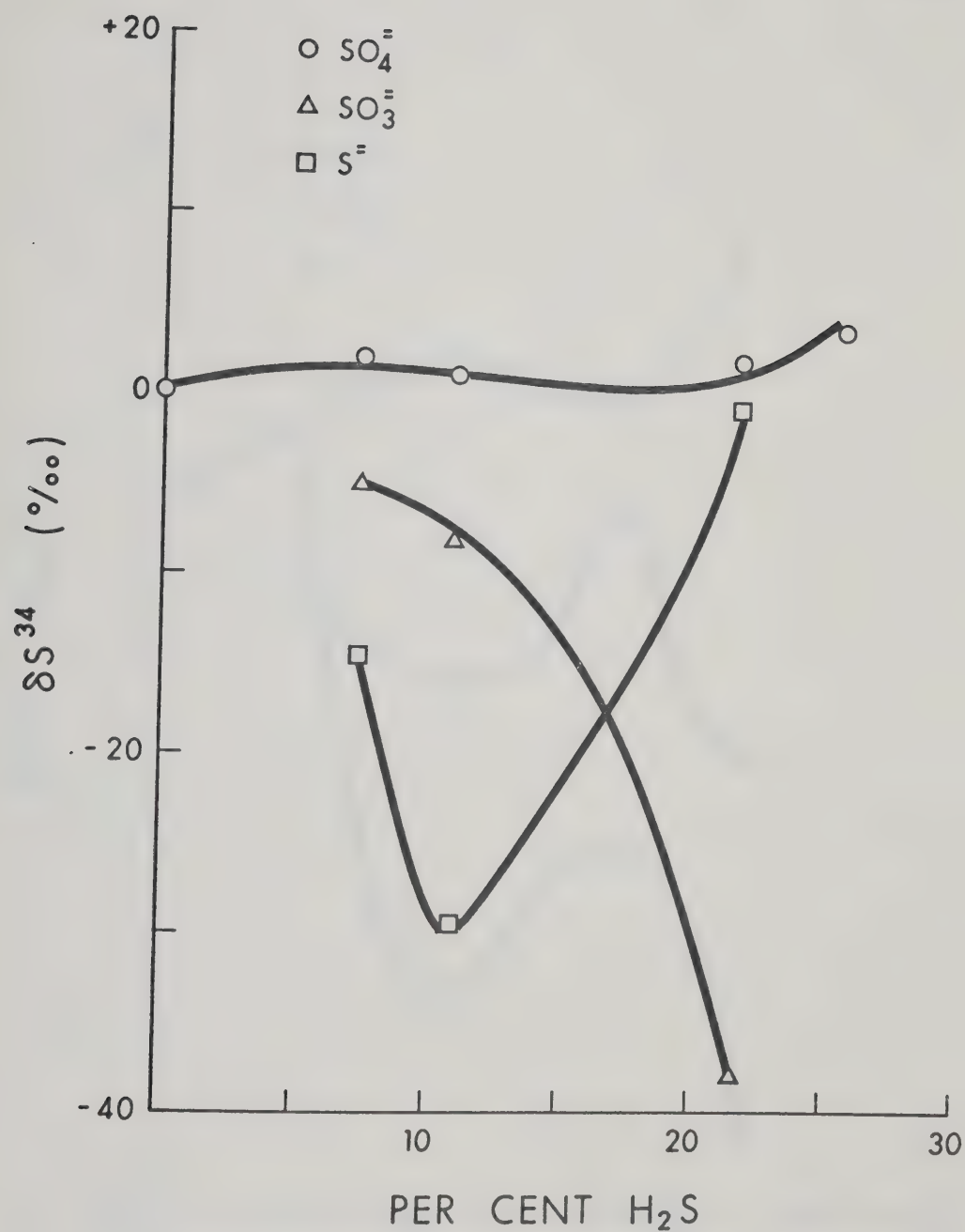


Figure 4-10 Isotope effects during bacterial
reduction No. 2 .

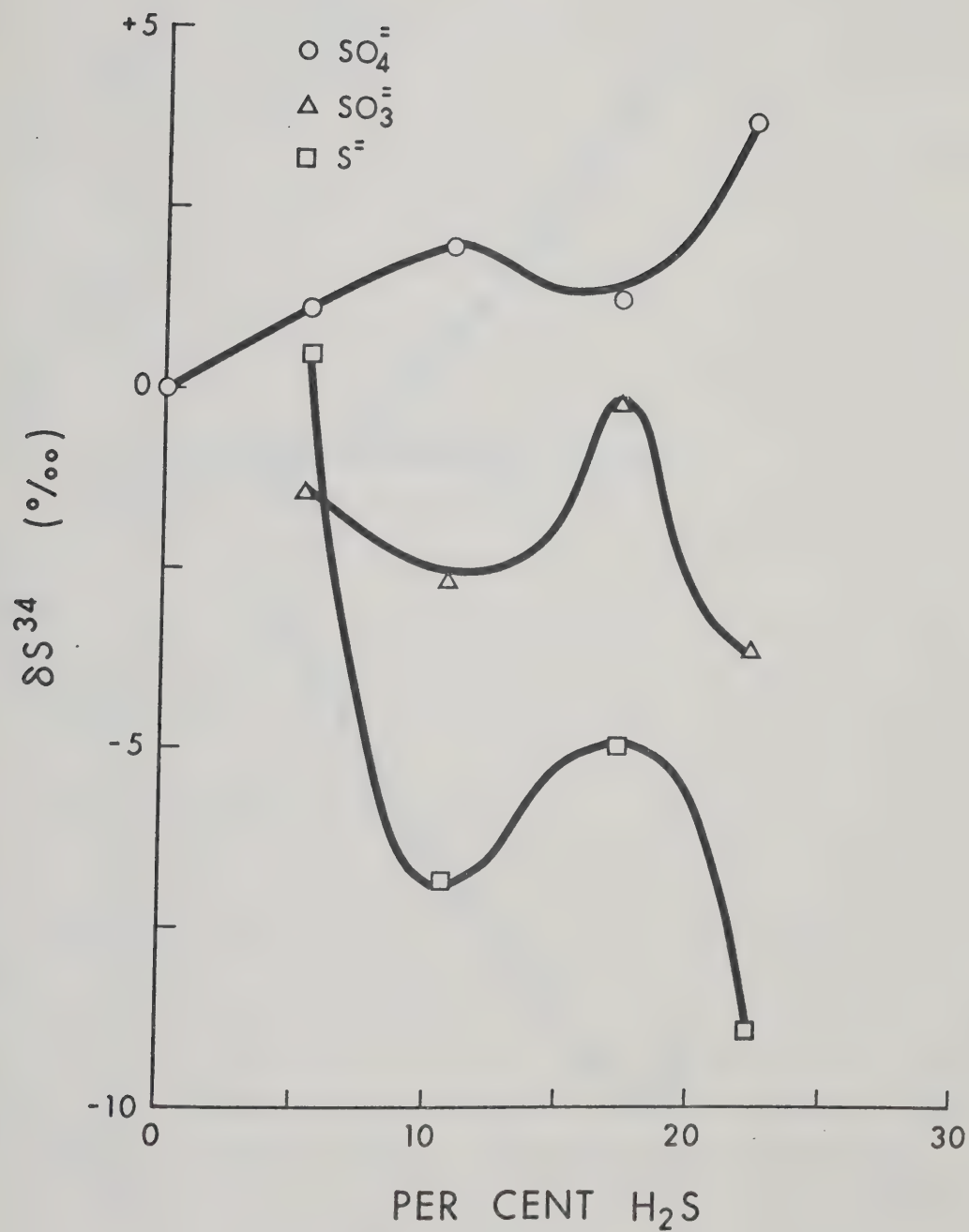


Figure 4-11

Isotope effects during bacterial
reduction No. 3 .

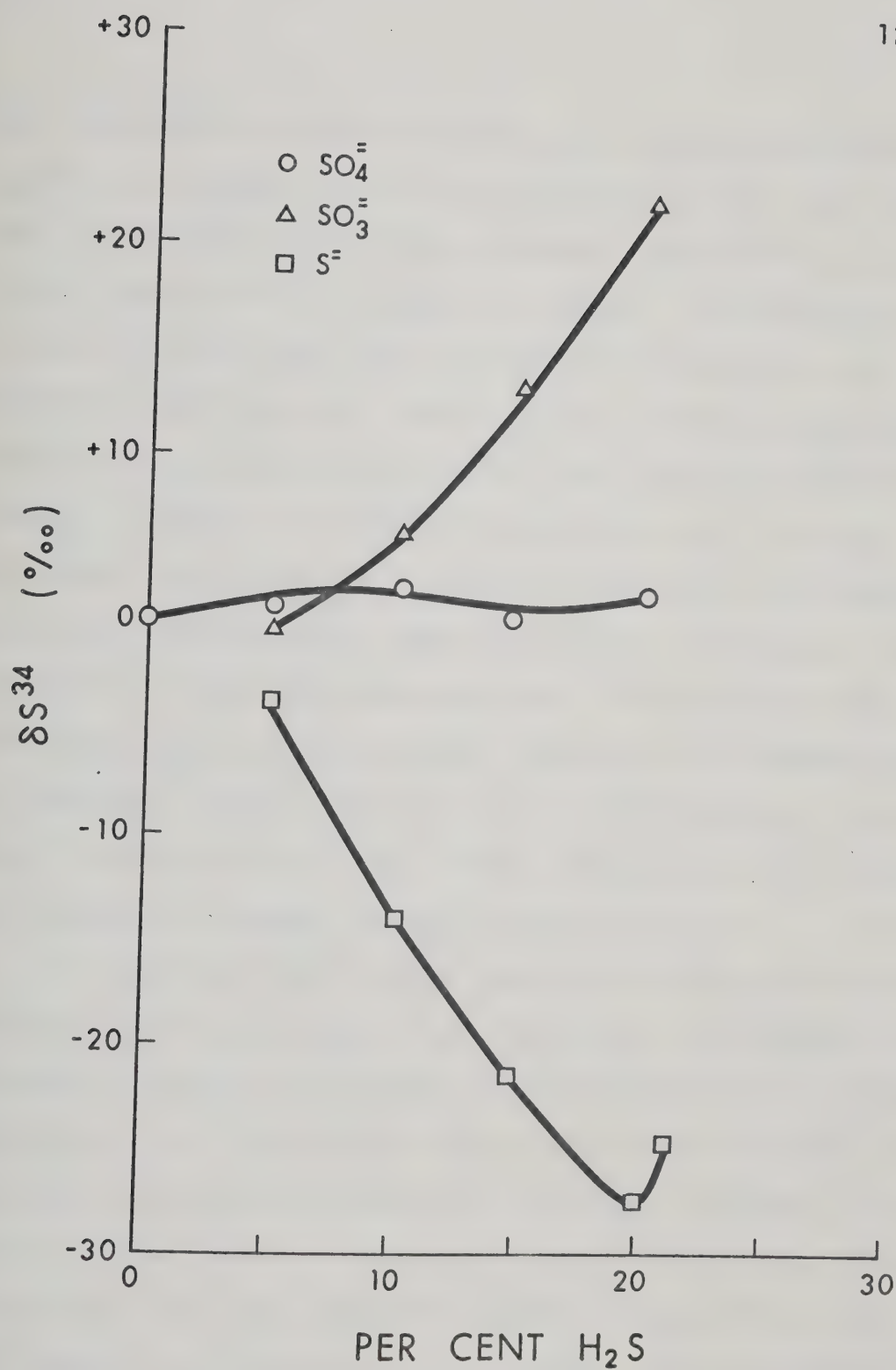


Figure 4-12

Isotope effects during bacterial
reduction No. 4 .

that the sulphite reservoir later stabilized as both the $\text{SO}_4\text{-SO}_3$ and $\text{SO}_3\text{-H}_2\text{S}$ reductions proceeded at about the same rate. The final H_2S fraction, however, implied that the $\text{SO}_4\text{-SO}_3$ reduction rate rapidly increased. This resulted in a large SO_3 reservoir where the sulphur-32 species were preferentially reduced. Thus a sudden increase in $\text{SO}_4\text{-SO}_3$ reduction would have accounted for the low δS^{34} value of the H_2S . However, it probably would also have produced sufficient quantities of sulphite to cause $\text{SO}_4\text{-SO}_3$ reduction cessation, since the SO_4 reducing bacteria are known to be sensitive to high SO_3 concentrations. Sulphite toxicity would have prevented any further reduction of sulphate, but not sulphite, unless there was either a deficiency in nutritional requirements or such a high concentration of sulphite, that even the sulphite reducers found it toxic.

Further verification was obtained from the percentage intermediate present at all stages of the reduction of sulphate. During this reduction experiment, the intermediate consisted of at least 27 per cent of the total sulphur atoms employed for sulphate reduction to hydrogen sulphide. Normally this was greater than 32 per cent, reaching as high as 39 per cent when the reduction ceased. Thus sulphite was not reduced as rapidly as it was formed. The accumulation of a large intermediate reservoir testified to predominant

SO_3 reduction rate control during the latter stages of the reduction. (Figure 4-7 had previously implied such rate control.) The general trends of run 3 (Figure 4-7) and MIZUTANI and RAFTER's (1969) run 1 at 14°C , seem to be similar. Their reduction only proceeded to 54 per cent reaction, in spite of the multiplicity of reducing organisms present. This could have resulted from an accumulation of intermediates, which were never reduced to H_2S . During run 3 of the present study, intermediates were always greater than 27 per cent of the total sulphur utilized. Such an accumulation during both reductions was believed to have caused rate control by the $\text{SO}_3\text{-H}_2\text{S}$ reducers. They were the only agents present that could remove the toxic sulphate from the reaction vessel. The general lightening of the H_2S product further suggested a modest sulphite reservoir, however the variations of both run 1 and 4 are difficult to explain. Table 4-3 shows that both these reductions had reasonably large intermediate reservoirs, however MIZUTANI and RAFTER's (1969) run 3 at 35°C had rather confusing variations (Figure 4-8) as well. Their 92 per cent reduction indicated some unreduced intermediate, but as with the present study, no meaningful conclusions were forthcoming. No rate controlling reduction was clearly obvious, but there existed a sufficiently large enough reservoir to have permitted $\text{SO}_3\text{-H}_2\text{S}$ isotopic selection.

A small sulphite reservoir seems to have been present during the earlier stages of run 2, however, Figure 4-10 shows that this reservoir was soon depleted, since the H_2S fractions became heavier. This would have been due to sulphite reduction occurring as fast as it became available, once the initial reservoir had been exhausted. Thus sulphite was reduced as rapidly as it was formed.

Further proof of a steadily decreasing sulphite reservoir can be seen in Table 4-3, where the per cent sulphite never exceeded 11 per cent of the total amount of sulphur atoms employed. Hence this reservoir was most precarious, threatening the survival of the sulphite reducing bacterial strain. These most adverse conditions suggested sulphate reduction rate control during the reduction of sulphate to the final H_2S product. MIZUTANI and RAFTER (1969) also observed such a rapid reduction of sulphite (run 2 at $25^{\circ}C$, Figure 4-8). Their 98 per cent reaction may have only been achieved because of the many reducing organisms employed in their study. Thus other pathways for reduction of sulphate to eventually sulphide may have occurred, but speculation on this point is most impractical.

Clearly, neither sulphate nor sulphite reduction could be solely responsible for rate control during the $\text{SO}_4 \rightarrow \text{SO}_3 \rightarrow \text{H}_2\text{S}$ reduction, rather a competition for control of the rate existed with some other factor determining which step would have controlled the $\text{SO}_4 \rightarrow \text{H}_2\text{S}$ bacterial reduction. This variable did certainly influence both the reduction rates, and in addition may have been responsible for the low yields observed with pure culture reduction experiments. Only two conclusions were possible, for such poor yields. Either the sulphate available for $\text{SO}_4\text{-SO}_3$ reduction had been depleted, or the available sulphate had not been entirely reduced to some intermediate.

If the sulphate available for reduction had been depleted, then such poor yields were a result of non-reduction of intermediates. If no sulphate was left, then intermediates such as SO_3 and S_2O_3 were unreacted and present in the reaction vessel. On the other hand, if there was sulphate remaining in the reaction vessel, then the poor yields resulted from a lack of completeness of the $\text{SO}_4\text{-SO}_3$ reduction.

Now if the sulphate available for reduction had been depleted, then the addition of BaCl_2 to the remanent media would have yielded no BaSO_4 precipitate. A few drops of HCl were added to the residual media then BaCl_2 in excess. The result was as expected. Precipitation of BaSO_4 was ob-

served, and the quantities were not negligible. The amount of unreacted sulphate was then determined to see if the cause of such poor yields could be explained. Exactly 100 ml of the remanent media and sulphate was used to evaluate quantitatively the amount of unreacted sulphate. The percentages of unreacted sulphur (in sulphate) in the remaining media were as follows.

Run 1 BaSO_4 = .73 mg/ml (S=100 mg/l) = 44.4% of initial
 Run 2 BaSO_4 = 1.15 mg/ml (S=158 mg/l) = 69.9% of initial
 Run 3 BaSO_4 = .98 mg/ml (S=135 mg/l) = 59.6% of initial
 Run 4 BaSO_4 = n. d. , accidentally discarded.

Thus there were large quantities of unreacted sulphate present throughout the reduction experiments. If this unreacted sulphate was ignored, then Figure 4-13 indicates the relative amounts of intermediate produced during each sulphate reduction experiment. The unreacted sulphate gave a further check on the amount of intermediate remaining once the reduction process ceased. (Previously the sulphate fractions were used to calculate the amount of intermediate present during the biological reduction of sulphate.) The amount of sulphate not reduced when metabolic processes ceased, indicated that the final amounts of intermediate were as follows.

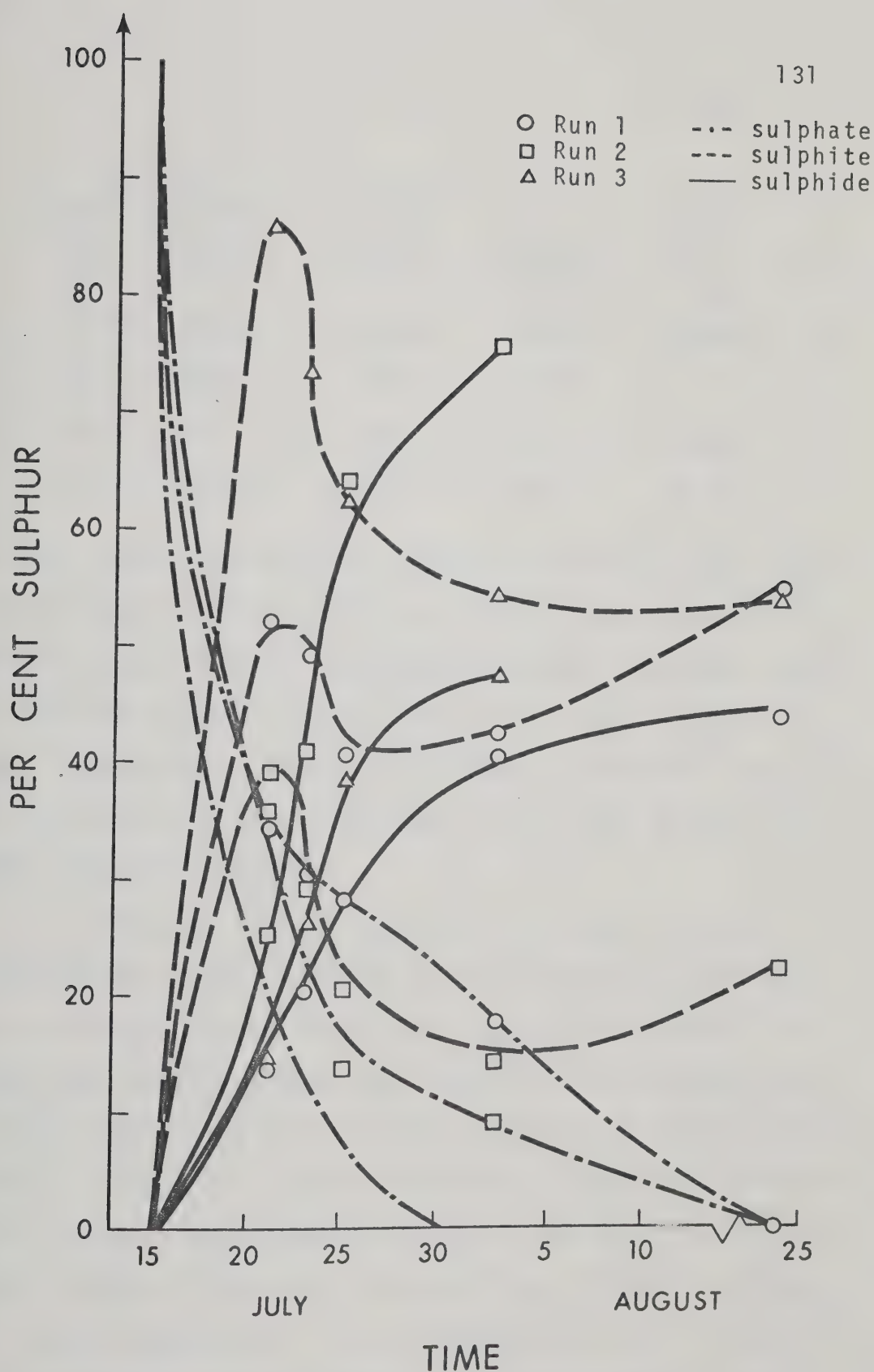


Figure 4-13. Sulphur distribution during the bacterial reduction of sulphate, ignoring unreacted sulphate remaining at the end of the conversion.

	<u>total H₂S</u>	<u>final un SO₄</u>	<u>un SO₄ removed</u>	<u>int. removed</u>	<u>final int.</u>
Run 1	24.4%	28.5%	21.8%	8.5%	16.8%
Run 2	22.7	45.0	27.9	2.0	2.4
Run 3	19.0	38.1	19.8	11.1	12.0
Run 4	19.1	n. d.	25.0	6.4	n. d.

Therefore, there was sulphate available for reduction, but it was not reduced even to sulphite. Within the limits of the chemical preparation and weighing techniques employed, this data confirms the rate control conclusions previously discussed. (Reduction 4 was not available for such an analysis, due to carelessness which resulted in the loss of the final sulphate and media sample.)

Although the per cent reduction was slightly modified by the temperature of the media and inoculum, the poor yields were caused entirely by some other factor. If sulphate was remaining when the reduction had ceased, then the reason would have been sulphite toxicity or simply some metabolic inadequacy. KAPLAN (1962) has stated that sulphite is toxic to most organisms by virtue of its ability to bind carboxyl groups of metabolic intermediates, thus preventing their further metabolism. Such a phenomenon could possibly have accounted for the cessation of microbiological activity in runs 1 and 4. If the reductions did not cease because of

sulphite toxicity, then the question arose as to why such a cessation occurred.

To keep the bacterial strains viable involved removing cultures to new media long before the media was believed to be exhausted of sufficient nutrients for good bacterial growth. Nearly 100 per cent reduction was observed by MIZUTANI and RAFTER (1969), but only when a conglomerate of unknown microorganisms were employed as found in stream mud. KEMP and THODE (1968), in an attempt to obtain greater isotopic fractionation, tried various bacterial strains and physiological states. However, their reductions with pure cultures did not exceed 26 per cent reaction. Complete reductions may be possible with a multitude of reducing bacteria and naturally occurring nutrients, but pure cultures have never escaped this fastidious nature of most microorganisms, when employed in laboratory experiments.

A probable reason for this microbial phenomena, and a possible explanation of why the sulphite reservoir was permitted to develop to such toxic levels, lay hidden in the mechanism involving rate control of the bacterial reduction. HARRISON and THODE (1958), KAPLAN and RITTENBERG (1964), and KEMP and THODE (1968), found the rate of reduction to be inversely proportional to the rate of isotopic fractionation, when lactate was used as the electron donor. However, two

such sulphate reduction experiments of KEMP and THODE (1968) were anomalous. These two reductions occurred at 11⁰ and 20⁰ C, and involved very slow sulphide production coupled with small isotope fractionation. This was attributed to a low temperature effect. What was most startling was that in one series of experiments, the rates of reduction and the isotopic enrichments were not reproducible. This was because there was a direct dependence on the reduction rate, rather than the inverse expected. MIZUTANI and RAFTER (1969) and the present study have also observed such phenomena. This was not a temperature effect, but rather some other factor was responsible for the variations observed.

HARRISON and THODE (1958) stated that S-O bond rupture was rate controlling if the fractionation factor was about 1.025. However, many investigations have since obtained larger values. Thus S-O bond rupture was not rate controlling. KAPLAN and RITTENBERG (1964) concluded that the rate controlling step involved the availability of electrons at the reduction site, hence rate control was dependent upon the electron donor. In the present study the same electron donor was used for all reductions, with the reduction rate and fractionation rate totally independent of the electron donor employed. The reduction rate was observed to be both directly and inversely proportional to the rate of

fractionation at different times during a single bacterial reduction of sulphate experiment. Such was the case for the investigations of MIZUTANI and RAFTER (1969) as well. Hence the rate controlling step during the reduction of sulphate was much more complicated than simple S-O bond rupture, electron donor, or temperature.

4.6 Conclusions

1. The rate of sulphite reduction was not always proportional to the rate of sulphate reduction. During a single microbiological reduction, both reduction processes were competing for control of the rate, with each rate controlling at various stages of a single $\text{SO}_4 \rightarrow \text{SO}_3 \rightarrow \text{H}_2\text{S}$ reduction.
2. Any accumulation of sulphite, during the bacterial reduction of sulphate, is toxic. Sulphite accumulation prevents complete sulphate reduction, because sulphite is toxic to most organisms by virtue of its ability to bind carboxyl groups of intermediates, thus reducing the rate of metabolism.
3. Meaningful microbiological conclusions were only believed possible when a pure culture and known media were employed, in spite of the fact that this is not characteristic of naturally occurring phenomena.
4. Pure cultures of bacteria cannot complete reduction of sulphate because of the simplicity of the media

employed. Only in nature, with its complex forms, nutrients, and pathways, might reduction possibly be complete. Strain acclimatization and poor yields pose the greatest problems in laboratory studies of pure cultures.

5. An important temperature independent relationship was observed between the O^{18}/O^{16} and the S^{34}/S^{32} ratios of the residual reactant sulphate. The relative enrichment of sulphur-34 to oxygen-18 was found to be approximately 4:1. (This will be discussed further in Sections 5.4 and 5.5.)

6. Large isotope fractionations were observed between the intermediate and the H_2S final product. In Run 1 the fractionation reaches as high as 70 per mil (unusually high, since MCCREADY (1974) recently obtained a large fractionation with Yeast of 50 per mil, which to date is considered very high).

Chapter V. Isotope Effects During Microbiological Sulphate Reduction

5.1 Microbiological Sulphate Reduction Mechanisms

In order to explain the observed isotope effects during microbiological sulphate reduction, it is necessary to examine mechanisms involved. The basis of these mechanisms is a redox reaction which occurs when an energy source becomes oxidized (loss of electrons) and another material becomes reduced (gain of electrons). Lactate was employed as the energy and carbon source, donating electrons, while the sulphate or sulphite ion accepted electrons during the oxidation - reduction experiments of the present investigation. KAPLAN and RITTENBERG (1964) assumed that this electron transfer was the rate controlling step during the microbiological reduction of sulphate.

Once the electron donor, the lactate, has been oxidized, it is no longer an energy source and may then serve as an electron acceptor. In such a situation, competition could occur between sulphate, sulphide, and the oxidized lactate, to serve as the electron acceptor.

The biological reduction of sulphate mechanisms are presented in Figure 5-1. These mechanisms are employed by most sulphate reducers. Although not all sulphate reducers belong to the genus *Desulphovibrio* or *Desulphatomatulum*, isolates from nature are most frequently species of these genera. These bacterial forms are obligate

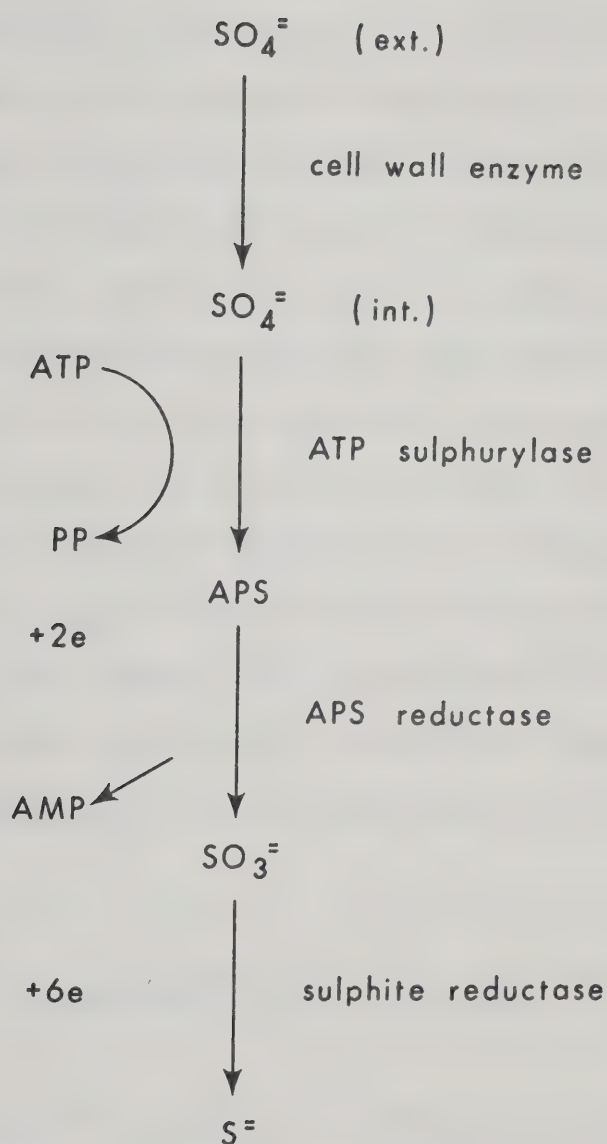
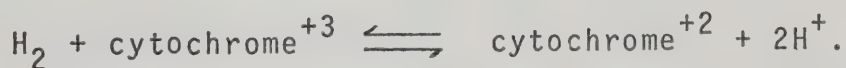


Figure 5-1 Mechanisms of bacterial sulphate reduction (dissimilatory).

anaerobes which can use either H_2 or organic compounds (such as lactate and ethanol) as the energy source.

During biological sulphate reduction, the energy source transfers electrons through a cytochrome system to the sulphate. This cytochrome is a natural electron carrier, which undergoes oxidation and reduction through a loss or gain of electrons from the iron atoms. These iron atoms were part of the culture media ($FeCl_2$) employed for the reduction experiments. In nature such a process plays a considerable role in the corrosion of iron and steel pipes. This cytochrome electron transfer is the first step of sulphate reduction. That is, with molecular hydrogen as the electron donor, this electron transfer is



(The cytochrome^{+3} is sometimes written as $\text{cytochrome } 2 Fe^{+3}$, and the cytochrome^{+2} as $\text{cytochrome } 2 Fe^{+2}$.)

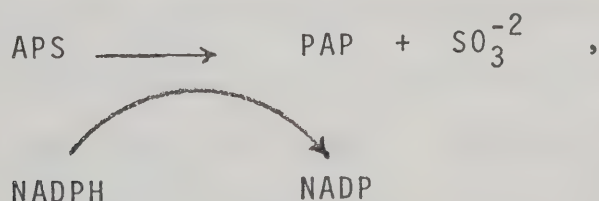
This transfer of electrons through a cytochrome system to the sulphate can only occur if the sulphate is in a form which can accept electrons. This is achieved by the high energy phosphorous compound ATP (adenosine triphosphate), which plays an important role in energy metabolism. The energy derived from oxidation-reduction

is conserved in the high energy bonds of the ATP. This energy is later released by the enzyme kinases for cell functions such as mobility, biosynthesis, and growth. The production of ATP occurs during substrate phosphorylation. This will occur only if a suitable electron acceptor is present. Such an electron acceptor is the sulphate ion.

In order to accept electrons, the sulphate ion must first be enzymatically converted to APS (adenosine phosphosulphate) in the presence of ATP. This is the second step of the reduction mechanism,

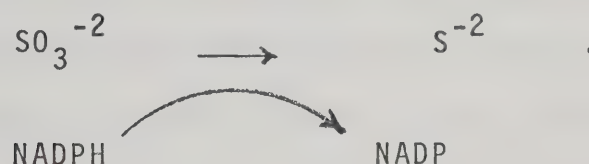


The product PP (pyrophosphate) forms when the sulphate radical is included in the APS. The sulphate ion is subsequently reduced to sulphite by the enzyme APS reductase. This third step can be represented as



where the APS reductase requires NADPH as an electron donor coenzyme.

The fourth step of the $\text{SO}_4\text{-SO}_3\text{-H}_2\text{S}$ reduction also involves an enzyme using NADPH as an electron donor. The NADPH coenzyme is not permanently attached to enzyme proteins, but rather combines with them only during the enzymatic reaction. This coenzyme NADPH (nicotinamide adenine dinucleotide phosphate) is an electron carrier which donates an electron, becoming the oxidized NADP. (NADPH is more accurately written as $\text{NADP} + \text{H}^+$). Thus the coenzyme NADPH is necessary in order that the sulphite reductase enzyme can reduce sulphite to sulphide. That is,



These four steps (see also Figure 5-1) are the known processes involved in anaerobic respiration. In such a reduction, the difference in mass of the isotopic species will be an important factor. Since energy, entropy, and the free energy of isotopic substances depend on the vibrational frequencies of the molecules, which in turn depend on the masses of the atoms in the molecules, it can be seen that there is a basis for the differences in properties of isotopic materials. Hence there will be different rates for each step of the reduction, favoring either the heavier or lighter isotopic species. Thus isotopic abundance ratios were believed able to elucidate which mechanisms were

rate controlling during the microbiological reduction of sulphate.

5.2 Reduction Mechanisms and the Isotopic Inversions

The mechanisms of biological sulphate reduction have been outlined (Section 5.1) as presented in BROCK (1970). KEMP and THODE (1968) discussed a similar pathway for the assimilation of sulphate, however, they noted two additional phases of sulphate metabolism which could give rise to isotope effects. They were, the assimilation of sulphate by the cell, and the passage of H_2S out of the cell. Thus the rate controlling step during $\text{SO}_4\text{-SO}_3\text{-H}_2\text{S}$ reduction should be one or more of the following:

- I. the assimilation of sulphate by the cell,
- II. the transfer of electrons at the reduction site,
- III. the assimilation of sulphate by ATP,
- IV. the reduction of APS to sulphite,
- V. analogous complicated enzymatic processes for the reduction of sulphite to sulphide, and
- VI. the passage of H_2S out of the cell.

To elucidate the rate controlling mechanisms of microbiological sulphate reduction, it became necessary to examine the isotopic data more closely. An explanation of the observed isotopic inversions (Figure 4.1) was thought to help in determining the rate controlling steps, since

both the sulphur and oxygen data indicated an isotopic inversion phenomena. However, interpretations are restricted since a chemostat was not employed for the bacterial reduction experiments. Thus the cell population and reduction pH were never known.

In such a closed system reduction experiment, the lactate electron donor was believed to have oxidized to acetic acid (CH_3COOH). With no pH regulation available, this sulphate reduction by-product would have lowered the pH of the culture media and inoculum. Since the enzymes associated with bacterial sulphate reduction are known to be very pH sensitive, a lowering of the inoculum pH would have caused cell death (lysis). The resulting intracellular sulphur accumulation would have permitted the remaining viable bacteria to metabolize these organic nutrients rather than the inorganic sources present in the culture media.

The intracellular sulphur present in dead cells has a lower δS^{34} value than the available sulphate (MCCREADY and DIN (1973)). Thus during culture regrowth on metabolites released from lysis, the sulphate isotopic composition would temporarily lighten. (Such an inversion was observed in Figure 4-4.) This regrowth metabolism, however, releases NH_4 , which would raise the inoculum pH. Once this regrowth had been established, the pH would increase, preventing further lysis. Once the metabolites released from lysis were exhausted, the inorganic sources

in the culture media would have been re-employed. The isotopic abundance data would then indicate a progressive enrichment of δS^{34} and δO^{18} in the sulphate. This was observed experimentally. Soon after this regrowth all microbiological activity ceased. This was thought to be a result of the increasing pH, preventing a viable enzymatic environment. This conclusion would support the known pH sensitivity of enzymes. Such an explanation for low yields is believed valid for similar sulphate reduction experiments where strict pH and population monitoring are not employed. Thus complete (100%) sulphate reduction is not believed possible unless these variables are controlled, such as can be done in a chemostat.

It should be noted that the intracellular sulphur released during lysis metabolism would not have produced a large isotopic enrichment, because of the limited intracellular reservoir. The present study data did possess isotopic enrichments of the same order of magnitude as expected (MCCREADY and DIN (1973)) from intracellular accumulations (i.e. less than 0.8 ‰ for sulphur and 0.2 ‰ for oxygen).

The isotopic inversions were believed caused by critical pH variations. No information concerning $SO_4-SO_3-H_2S$ reduction rate control was thought possible from an examination of the isotopic inversions. Hence pH contributed

to rate control only in so far as it regulated cell populations and isotopic enrichments. The pH variations were also believed to be responsible for some of the very large fractionation factors observed during bacterial reduction of SO_4 experiments. This will be discussed later in Section 5.4.

The isotopic data of Section 5.4 indicate that the bacterial reduction of sulphate was strictly a first order kinetics reaction. The fractionation factors (α) were consistent with first order kinetics, but the fraction fractionation factors (β) certainly were not. REES (1973) has recently discussed isotopic fractionation effects during the reduction of sulphate to hydrogen sulphide in experiments with *Desulphovibrio desulphuricans*. He has presented a steady state model which explains higher fractionation factors in terms of full reversibility of flows between the external sulphate and the internal sulphite. This model will help to explain the high fractionation factors of the present study, but it involves the addition of back reactions to the forward reactions presented in Section 5.1 as possible rate controlling steps during bacterial sulphate reduction. Thus these high fractionation factors may involve reversibility of the mechanisms of biological sulphate reduction, but they yield no additional possibilities for elucidation of the rate controlling steps. Therefore the forward reaction steps must be modified

to include full reversibility, with the additional steps being:

- VII. the internal sulphate accumulation and a back reaction to external sulphate,
- VIII. the accumulation of APS and a back reaction to ATP liberating the sulphate ion,
- IX. a sulphite accumulation and a back oxidative reaction to APS,
- X. an internal H_2S accumulation resulting in the back oxidative reaction to sulphite, and
- XI. an accumulation of H_2S in solution which re-enters the bacterial cell.

The possible steps involved in biological sulphate reduction are believed complete. It is now necessary to discuss each step in terms of known experimental evidence, to determine which steps are rate controlling.

5.3 The Rate Controlling Steps

An attempt was made by HARRISON and THODE (1958) to explain the mechanisms controlling fractionation during sulphate and sulphite reduction. They concluded that the breaking of the S-O bond during the reduction of sulphate to sulphite, was the dominant process controlling fractionation at low rates of sulphate reduction. This S-O bond rupture was believed to possess a kinetic isotope effect

of 22 ‰, which does not adequately explain more recent data where much greater fractionations have been observed in both laboratory experiments and in nature.

The temperature, sulphate concentration, electron donor, and pH, within the normal physiological range of these parameters, were concluded to influence fractionation only in so far as they influenced the rate of reduction (KAPLAN and RITTENBERG (1964), and KEMP and THODE (1968)). Thus it was concluded that the rate controlling step must be one or more of the forward or backward reaction steps previously outlined.

The initial step (Step I) of the bacterial reduction process is the uptake of sulphate by the bacteria. This process is first order with respect to sulphate at low sulphate concentrations (HARRISON (1957)). At higher concentrations, the rate of sulphate uptake is independent of the sulphate concentration. Here the limiting factor is most probably the supply of an enzyme which complexes with the sulphate ion to transport it across the bacterial cell wall. REES (1973) treated this step as zeroth order with respect to sulphate, and postulated the establishment of steady state conditions at such concentrations. If the rate of entry becomes rate controlling, then no matter what isotope effects are possible in the reduction phase of metabolism, the isotope effects observed will be only those due to entry of the oxidant.

The bacterial uptake of sulphate was concluded by KAPLAN and RITTENBERG (1964), KEMP and THODE (1968), and REES (1973) to have a negligible isotope effect. If the rate of entry of sulphate into the cell was rate controlling, then at low reduction rates the fractionation would have been almost zero. This is not in agreement with experimental evidence, since with lactate or ethanol as the electron donor, the fractionation rate was observed to be inversely proportional to the rate of reduction. REES (1973) noted that this first forward Step I possessed an inverse isotope effect of -3‰ , so that S^{34}O_4 uptake is favored over S^{32}O_4 uptake. He also concluded that the isotope effects in either the forward (Step I) or backward (Step VII) steps involving SO_4 uptake, were probably small since they are associated with reactions where the oxidation state of sulphur is not altered.

Hence Step I and Step VII of the reaction sequence were concluded to be not rate controlling, except at low sulphate concentrations. These processes, as concluded by REES (1973), were then zeroth order with respect to sulphate concentration. On the other hand, the passage of H_2S from the cell is presumably fast and hence not rate controlling since H_2S is toxic towards cell constituents. REES (1973) concluded that Step VI, the passage of H_2S from the cell, involved only a small isotope effect and

was not rate controlling. He also concluded that the backward Step XI possessed a small isotope effect and was similarly not rate controlling. Thus the rate controlling steps are to be found during the reduction of sulphate and/or sulphite in the bacterial cell.

Steps V and X involve analogous but more complicated enzymatic processes than are involved in $\text{SO}_4 \rightarrow \text{SO}_3$ reduction. The present study (Section 4.5) and the investigation of MIZUTANI and RAFTER (1969) have shown that the rate of controlling step occurred in both $\text{SO}_4 - \text{SO}_3$ and $\text{SO}_3 - \text{H}_2\text{S}$ reduction, hence it must have involved some biochemical process common (analogous) to each.

The bacterial reduction of Sulphate solely by a sulphate reducer as *Desulphovibrio desulphuricans* did not permit other investigators to realize that rate control was common to both $\text{SO}_4 - \text{SO}_3$ and $\text{SO}_3 - \text{H}_2\text{S}$ reductions.

Although detailed biochemical mechanisms of sulphite reduction are not known, it is assumed that a similar enzymatic pathway should have been the reduction mechanism for $\text{SO}_3 - \text{H}_2\text{S}$ reduction. This would have been somewhat more complicated in that three oxygen atoms are removed as compared to only one in the $\text{SO}_4 - \text{SO}_3$ reduction. Regardless, both reduction processes were observed to be rate controlling when all other parameters apparently were constant. Hence the rate controlling step was not sulphate

nor sulphite reduction, but rather some bacterial process common to both reductions. Thus Steps V and X can be eliminated from the list of possible rate controlling steps.

It should be noted that the poor yields of other investigators and the present study were most probably a result of the experimental media employed. MIZUTANI and RAFTER (1969) and NAKAI and JENSEN (1964) obtained good yields employing media found in nature, since these materials are much more complex than simple laboratory preparations. Also the electron donors used in previous investigations were not materials found commonly in nature (lactate itself is not so abundant in nature). Acclimatization of the bacteria to these artificial nutrients was then a possible explanation for the poor yields. KEMP and THODE (1968) found strains that would never acclimatize to laboratory media. Hence the apparent fastidious nature of microorganisms in laboratory experiments may have been solely a result of poor medium acclimatization, poor choice of electron donor, enzymatic and cell deficiencies in the media, and difficulty in microbiological utilization of non-natural energy sources.

Actual rate control may be linked to a poor acclimatization of simplified media constituents and inorganic energy sources. Furthermore, the detailed biochemical

mechanisms of energy source reduction by sulphate and sulphite reducers is still not fully understood. The $\text{SO}_4 - \text{SO}_3$ reduction process has been investigated as outlined previously from BROCK (1970). KEMP and THODE (1968) mentioned specific details of this reduction that were noted in BROCK (1970), but were the results of investigations by POSTGATE (1956), ISHIMOTO, KOYAMA, and NAGAI (1954), ISHIMOTO (1959), and PECK (1959, 1960, 1961, 1962). At present the enzymatic requirements involved are not understood well enough to permit good laboratory experiments to be carried out (i.e. 100 % reduction of available sulphate).

If enzymatic requirements are the basis for the poor yields reported, then only further microbiological investigations will clarify this lack of understanding concerning the microbiological reduction of sulphate and sulphite. If poor acclimatization by the bacterial strains is not rate controlling, then the possible steps that could have controlled the reduction rate should be:

- II. the transfer of electrons at the reduction site,
- III. the assimilation of sulphate by ATP,
- IV. the reduction of APS to sulphite, and
- IX. a sulphite accumulation involving a back oxidative reaction to APS.

Extensive study is still needed to completely understand rate control during microbiological sulphate reduction, and isotope studies may not be solely capable of providing all the solutions. However, isotope data can be analyzed to yield information about some of the physical processes involved.

5.4 Isotope Effects

When a substance continuously loses material that had a fixed isotopic ratio relative to that of the remaining substance, the Rayleigh distillation equation is applicable. To express the change in isotopic ratio in terms of the " δ " notation, the Rayleigh equation

$$\delta - \delta_0 = 1000 (\alpha - 1) \ln F$$

is employed. Here δ_0 is the isotopic composition of the original sulphate, δ is the isotopic abundance ratio of the sulphate when the fraction of sulphate remaining is F , and α is the average kinetic fractionation factor for this process. The enrichment factor $1000(\alpha-1)$ was calculated for both sulphur and oxygen isotopes in the sulphate ion. The results appear in Table 5-1. The ratio of the sulphur-34 enrichment to the oxygen-18 enrichment in the sulphates was found to be approximately 4:1. That is, the enrichment re-

Calculation of the kinetic fractionation factors (α)
for oxygen and sulphur isotopes in sulphate during
the bacterial reduction of sulphate

Table 5-1

Specimen Number	Sulphate ($\delta S_t^{34} - \delta S_o^{34}$)	($\delta O_t^{18} - \delta O_o^{18}$) ($^{\circ}/_{\text{oo}}$)	Fraction F	-ln F	1000($\alpha-1$) S	1000(α_s-1) 1000(α_o-1)
1 - 0	0.00	0.00	1.000	0.000	---	---
1 - 1	2.16	0.58	.642	.443	4.9	1.3
1 - 2	2.02	0.47	.619	.480	4.2	1.0
1 - 3	1.98	0.44	.606	.501	4.0	0.9
1 - 4	2.65	0.66	.549	.600	4.4	1.1
1 - 5	3.73	0.90	.442	.816	4.6	1.1
					22.0	5.4
2 - 0	0.00	0.00	1.000	0.000	---	---
2 - 1	1.93	0.46	.819	.200	9.7	2.3
2 - 2	1.33	0.32	.797	.227	5.9	1.4
2 - 3	2.45	0.69	.752	.285	8.6	2.4
2 - 4	3.84	1.02	.735	.308	12.5	3.3
					36.6	9.4
3 - 0	0.00	0.00	1.000	0.000	---	---
3 - 1	1.11	0.26	.553	.592	1.9	0.4
3 - 2	2.16	n. d.	.563	.574	3.8	n. d.
3 - 3	1.37	0.33	.540	.616	2.2	0.5
3 - 4	3.34	0.86	.549	.600	5.6	1.4
					9.6	2.4
4 - 0	0.00	0.00	1.000	0.000	---	---
4 - 1	1.35	0.33	.717	.333	4.1	1.0
4 - 2	1.94	0.49	.690	.371	5.2	1.3
4 - 3	1.15	0.29	.708	.345	3.3	0.8
4 - 4	1.58	0.45	.664	.409	3.9	1.1
					16.5	4.3
					22.03	4.10
					5.37	
					36.58	3.88
					9.44	
					9.62	3.99
					2.41	
					16.47	3.87
					4.25	
					avg.	3.96

lationship between the two kinetic fractionation factors was calculated to be

$$\frac{\alpha_s - 1}{\alpha_0 - 1} = 3.96 \quad .$$

The kinetic fractionation factors (α) calculated using the Rayleigh distillation equation, were average fractionation factors for the bacterial reduction process. The relative enrichment ratio of the two isotopic species was in agreement with that observed by MIZUTANI and RAFTER (1969) during their independent but concurrent investigation. In spite of the differences in media, organisms, and techniques of both studies, this 4:1 ratio remained relatively independent of all factors save the presence of sulphate reducing bacteria. An extensive analysis of their data appeared somewhat out of order because of their employment of several flasks. However, since with pure cultures (present investigation) a similar phenomena was observed, it seemed reasonable to analyze their observations further.

The kinetic (average) fractionation factors (α) of both studies yielded enrichment ratios of about 4:1. The fraction fractionation factors (β) for each sulphate fraction removed did not appear to follow the trend of the kinetic fractionation factors (α). The fraction fractionation factors (β) were calculated for both investigations as follows.

Sample 1-1 removed at 35.8% reaction, $\alpha_s = 1.0049$

$$\alpha_o = 1.0013$$

Sample 1-2 removed at 38.1 reaction, $\alpha_s = 1.0042$

$$\alpha_o = 1.0010$$

For this period, the sulphur β_{1-2} was evaluated using the relationship

$$(\beta_{1-2})(\% \text{ reacted}) = [(\% \text{ reaction})(\alpha_{1-2})] - [(\% \text{ reaction})(\alpha_{1-1})] ,$$

where numerically for the interval under examination

$$(\beta_{1-2})_s (38.1 - 35.8) = (38.1)(1.0042) - (35.8)(1.0049)$$

$$2.3 (\beta_{1-2})_s = 38.260 - 35.975 = 2.285$$

$$(\beta_{1-2})_s = \frac{2.285}{2.3} = 0.9935 .$$

Similarly for the oxygen isotope fractionation,

$$(\beta_{1-2})_o = \frac{38.137 - 35.847}{2.3} = 0.9957 .$$

For the designated interval, the resultant enrichment factor then became

$$\frac{(\beta_{1-2} - 1)_s}{(\beta_{1-2} - 1)_o} = 1.50 .$$

The fraction fractionation factors (β) for the bacterial reduction of sulphate experiments are recorded in Table 5-2. The ratio of the sulphur to oxygen isotopic enrichment yielded values much removed from the consistent 4:1 observed with the kinetic fractionation factors (α). The data of MIZUTANI and RAFTER (1969) also was investigated and similar results were noted (Table 5-2). Both studies yielded varying enrichment ratios, but the mean was approximately 4:1. These fraction fractionation factors are expected to give wider fluctuations than the kinetic fractionation factors because of the accumulation of random errors involved in their calculation. Of interest, is that even the wildest fraction fractionation factors are consistent with the 4:1 ratio.(Apparently first order kinetics are not applicable unless just the kinetic fractionation factors are considered. Recently REES (1973) concluded that such bacterial reductions were not first order. This will be discussed more fully in Section 5.2.) The fractionations observed in the present investigation will also be discussed later (Section 5.2), and explained in terms of non-microbiological fractionation arising from enzyme mediated chemical equilibrium.)

The relative isotopic enrichment observed during the bacterial reduction of sulphate was calculated to be

about 4.0. The isotopic data as presented in Figure 4-5 confirmed this ratio. The relative enrichment observed was not unique. The oxygen and sulphur isotopic composition of sulphate from water in Lake Vanda, Antarctica, was reported by RAFTER and MIZUTANI (1967b) to possess such an enrichment of the isotopes. A plot of their δS^{34} values for the sulphate against the δO^{18} values yielded an approximate straight line of slope 4.0. At depth, this lake consisted of strongly density-stratified non-convective saline water. RAFTER and MIZUTANI (1967b) assumed that the enrichment was due to biological fractionation. (Biological activity was known to exist in this lake.)

MIZUTANI and RAFTER (1969), as previously mentioned, conducted three biological reduction of sulphate experiments employing sulphate free wet stream mud as the inoculum. In each case, they found the sulphur-34 enrichment of the sulphate to be about four times the oxygen-18 enrichment. Also, this ratio was observed to be independent of the temperature of the reduction. No explanation was given as to why this relative enrichment ratio was approximately four, nor was any attempt made to determine the microbiological activity in the stream mud inoculum.

The results of the present study confirmed the relative enrichments observed by MIZUTANI and RAFTER (1969).

However, this confirmation came from four most unusual sulphate reduction experiments. The inversions were decidedly independent of the kinetic or fraction fractionation factors, and the isotopic compositions of the sulphate in Figure 4-5 showed that the inversions only produced changes of direction along the "iso-enrichment" slope of 4:1. The relative enrichment of the unreacted sulphate remained constant, whether enriched or depleted in sulphur-34 or oxygen-18. Thus the biological reduction of sulphate was assumed to always follow such an "iso-enrichment" process, with the reduction demanding sulphur and oxygen isotopic correlation with respect to the relative enrichment of both isotopic species.

5.5 The 4:1 Relative δS^{34} to δO^{18} Enrichment

The 4:1 relative enrichment ratio of the sulphur and oxygen isotopic compositions was most intriguing. A simple explanation involves the initial S - O bond rupture.

The enrichment in sulphur - 34 of the unreacted sulphate was observed to be four times that of the oxygen - 18 . For the initial S - O bond rupture, there is a choice of four oxygen atoms in a given sulphate ion; however only one sulphur atom is involved. Thus simple statistics suggests a per atom enrichment in sulphur to be four times as great as that for the oxygen. This simple argument does not consider bond energies which are identified on a theo-

retical basis with kinetic isotope effects.

This relative enrichment ratio should also be able to be predicted theoretically, since reaction rate constants are different for competitive reactions of isotopic molecules. This can be done from a statistical point of view, using the theory of "absolute rates". The ratio of the rate constants of two isotopic molecules can be expressed as a simple function of the vibrational energy levels of the two molecules. BIGELEISEN and MAYER (1947) have approximated this ratio of the rate constants (κ) to be;

$$\frac{\kappa_1}{\kappa_2} = \frac{s_1}{s_2} \cdot \frac{s_2^\dagger}{s_1^\dagger} \cdot \frac{k_1}{k_2} \cdot \left(\frac{m_2^*}{m_1^*} \right)^{1/2} \cdot \left(1 + \right.$$

$$\left. \sum_i^{3n-6} G(u_i) \Delta u_i - \sum_i^{3n'-6} G(u_i^\dagger) \Delta u_i^\dagger \right) ,$$

where , s = the symmetry number of the reactants,

s^\dagger = the symmetry number of the activated complex,

k = the transmission coefficient,

m^* = the effective mass,

$u = h\nu/KT$,

ν = the vibrational frequency of the molecule, and

$$G(u) = 1/2 - 1/u + 1/(e^u - 1) .$$

The difficulty in utilizing the Bigeleisen - Mayer relation rests in the lack of information concerning the activated complex. One also has the problem of deciding a value for the effective mass. In practice, various approximations are made (REES and THODE (1965)) to see which theoretical model best fits the experimental data.

The simplest model to consider is that where the reaction is approximated by a simple S - O rupture. Two cases can be considered. The activated complex is the completely dissociated molecule, in which case

$$\sum_i^{3n^+ - 6} G(u_i^+) \Delta u_i^+ = 0 \quad (\text{Case I}) .$$

The second possibility is that the activated complex is similar to the reactant S - O bond, in which case the kinetic isotope effect is simply given by the effective mass term (Case II). In both situations, different effective mass terms may be tried, such as S - O (atoms) and SO₃ - O (fragments).

Since G(u) has been tabulated by BIGELEISEN and MAYER (1947) as a function of u in a convenient form for rapid calculation, the ratios of the rate constants for isotopic substitution in these two cases can be evaluated. The calculations are summarized for each case as follows.

<u>RATIO</u>	<u>CASE I</u>		<u>CASE II</u>	
	<u>Atoms</u>	<u>Fragments</u>	<u>Atoms</u>	<u>Fragments</u>
$\frac{K_{32}}{K_{34}}$	1.02695	1.01891	1.00994	1.00204
$\frac{K_{16}}{K_{18}}$	1.10645	1.11772	1.03931	1.04990
$\frac{K_{32}}{K_{34}} - 1$	0.253	0.161	0.253	0.041
$\frac{K_{16}}{K_{18}} - 1$				

(All data (vibrational frequency, etc.) from HERTZBERG.)

Thus it can be seen that the 4:1 ratio of $\delta S^{34}:\delta O^{18}$ is not predicted by a simple S - O rupture model. Indeed, the relative enrichments , as shown in the last line of the above data, of sulphur - 34 and oxygen - 18 are reversed.

This whole problem is further complicated by the recent findings of MIZUTANI and RAFTER (1973) , where the δO^{18} value of the sulphate remaining in the bacterial reduction was observed to depend upon the δO^{18} value of the water in which the sulphate was reduced by the bacteria. They interpreted this as an oxygen isotope exchange between the sul-

phate oxygen and the water through intermediates in the bacterial reduction of sulphate.

The laboratory experiments of MIZUTANI and RAFTER (1973) involved both mixed and purified cultures. Extrapolation of their findings yields $\delta\text{H}_2\text{O}^{18}$ values which would give 4:1 relative enrichments, however, the corresponding $\delta\text{H}_2\text{O}^{18}$ values differ for each experiment and range widely (+12 ‰ to -10 ‰). The implications of their work seem to suggest that the 4:1 ratio does not have particular significance. However, at this time the 4:1 observation should not be so readily dismissed. The work of MIZUTANI and RAFTER (1973) cannot adequately explain why so many workers have found the 4:1 ratio in laboratory experiments (LLOYD (1968), MIZUTANI and RAFTER (1969), the present study) and in terrestrial situations (MIZUTANI and RAFTER (1967), MIZUTANI and RAFTER (1969)).

No doubt, the fact that their reductions were carried out for up to 89 days was a factor in promoting oxygen isotope exchange, and can account to some extent for the difference between their work and that of others. However, this argument is not the limitation since the 4:1 ratio has been observed terrestrially.

The study of MIZUTANI and RAFTER (1973) did not follow first order kinetics. Their data indicated that back oxidative reactions do occur, and are an integral part

of biological sulphate reduction. This was also reported by TRUDINGER and CHAMBERS (1973) using S^{35} label. Thus the pathways available for $SO_4 \rightarrow SO_3 \rightarrow H_2S$ reduction are many, and the role played by the intermediates and back oxidative reactions has yet to be fully understood. The relative enrichment of $\delta S^{34} : \delta O^{18}$ has to depend upon the physical and chemical effects involved in both the forward and backward reactions.

It is hard to believe that the 4:1 relative enrichment ratio does not have special significance when in this thesis, it was encountered persistently during both normal and inverse kinetic isotope effects.

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APPENDIX

PDP 8 PROGRAM LISTING

/ COMMONLY USED VARIABLES AND SUBROUTINES .

*30

0030	0000	INTIME,	0000
0031	0000	GMIIME,	0000
0032	0000	XORSOR,	0000
0033	0520	WRITE,	NWRITE
0034	0200	CHAR01,	LIST01
0035	0213	CHAR02,	LIST02
0036	0226	CHAR03,	LIST03
0037	0243	CHAR04,	LIST04
0040	0271	CHAR05,	LIST05
0041	0305	CHAR06,	LIST06
0042	0321	CHAR07,	LIST07
0043	0357	CHAR10,	LIST10
0044	7775	M3,	-3
0045	0003	K3,	3
0046	0722	CRLF,	NCRLF
0047	0730	TYPE,	NTYPE
0050	0550	LISN,	NLISN
0051	0534	BELL,	NBELL
0052	0560	EXPON,	NEXPON
0053	0740	TAB,	NIAB
0054	0000	GMCT,	0000
0055	0400	TRIAPT,	TRIPAD
0056	1200	DOUBPT,	DOUBLE
0057	0441	TRIDIV,	TRIPDV
0060	0061	OLXIPT,	OLXI
0061	0000	OLXI,	0000
0062	0000	OLHI,	0000
0063	0000	OLLO,	0000
0064	5200	STORE,	5200
0065	0600	DECPRT,	SDPRNT
0066	0067	GMPT,	GMHI
0067	0000	GMHI,	0000
0070	0000	GMMED,	0000
0071	0000	GMLOW,	0000
0072	0000	TEMPTM,	0000
0073	0000	TEMTM,	0000
0074	0000	TEMTN,	0000
0075	0000	TEMTO,	0000
0076	0000	TEMTP,	0000
0077	0000	TEMTO,	0000
0100	0000	TEMTR,	0000
0101	0000	TEMTS,	0000
0102	0000	TEMTH,	0000
0103	4200	LOCTON,	4200
0104	4600	LOCTEN,	4600

0105	0000	NUMT,	0000
0106	0000	CNTR,	0000
0107	0000	QUOT1,	0000
0110	0000	QUOT2,	0000
0111	0000	DVSOR1,	0000
0112	0000	DVSOR2,	0000
0113	0000	DV1,	0000
0114	0000	DV2,	0000
0115	0000	DV3,	0000
0116	0000	DV4,	0000
0117	0113	DVDPT,	DV1
0120	0111	DVSPT,	DVSOR1
0121	2000	DMULPT,	DMUL
0122	2200	DBDVPT,	DUBDIV
0123	0124	TEN5PT,	TEN5
0124	0030	TEN5,	30
0125	3240		3240
0126	0000	ADD,	0000
0127	0107	QUOTPT,	QUOT1
0130	1000	RDVMPPT,	READVM
0131	1611	RATAGN,	AGNRAT
0132	2141	DV2PT,	2141
0133	1130	SETIS,	TSETIS
0134	1600	RATIOS,	RATIO
0135	0756	DEPOST,	DEPXOS
0136	4040	MEANNF,	NMEANS
0137	1400	DVDRTO,	DVDRAT
0140	1676	STDDEV,	STDDVN
0141	0752	DPCHAG,	DEPCHG
0142	1707	STDTW,	STDTWO
0143	2400	INCASE,	TWCASE
0144	1461	SQRT,	SQRTNO
0145	1441	HAPPY,	HAPPYS
0146	2511	AGAIN,	AGAIN3
0147	2537	STXORS,	STXOS
0150	2624	ZERDEC,	DECZER
0151	2633	ZTYPE,	ZRTYPE
0152	3016	GOON,	ONGO
0153	2600	CNTR0,	CNTR0S
0154	2711	LINE,	LINES
0155	0000	TABCNT,	0000
0156	2671	DVONE,	ONEDV
0157	3051	NEGT,	NEGIT
0160	2142	R1CNT,	2142
0161	2143	R2CNT,	2143
0162	2731	XOSMB,	MBXOS
0163	0266	SAYLCN,	266
0164	0000	NEWXOS,	0000
0165	3000	DEVSET,	SETDEV
0166	3014	BLAH,	3014
0167	0000	EXTRA,	0000

0170	2747	KORECT,	CORECT
0171	1131	BACKST,	1131
0172	1334	STAR70,	STARS
0173	1673	CNTXOS,	XOSCT
0174	3200	DELETE,	DELEIT
0175	3400	MUMCRT,	CRTMUM
0176	3500	TAB50,	TABAS0
0177	3550	NUMTCR,	CRNUMT

/ MESSAGES TO BE PRINTED

*200

0200	0200	LIST01,	.	/ -12 (-10 DEC)
0201	0311		311	/ INTIME=2**
0202	0316		316	
0203	0324		324	
0204	0324		324	
0205	0315		315	
0206	0305		305	
0207	0275		275	
0210	0262		262	
0211	0252		252	
0212	0252		252	
0213	0213	LIST02,	.	/ -12 (-10 DEC)
0214	0307		307	/ GMTIME=2**
0215	0315		315	
0216	0324		324	
0217	0311		311	
0220	0315		315	
0221	0305		305	
0222	0275		275	
0223	0262		262	
0224	0252		252	
0225	0252		252	
0226	0226	LIST03,	.	/ -14 (-12 DEC)
0227	0324		324	/ THIS SET IS
0230	0310		310	
0231	0311		311	
0232	0323		323	
0233	0240		240	
0234	0323		323	
0235	0305		305	
0236	0324		324	
0237	0240		240	
0240	0311		311	
0241	0323		323	
0242	0240		240	
0243	0243	LIST04,	.	/ -25 (-21 DEC)
0244	0306		306	/ FIRST SET MUST BE S !
0245	0311		311	
0246	0322		322	
0247	0323		323	
0250	0324		324	
0251	0240		240	
0252	0323		323	
0253	0305		305	
0254	0324		324	
0255	0240		240	
0256	0315		315	
0257	0325		325	
0260	0323		323	

0261	0324	324	
0262	0240	240	
0263	0302	302	
0264	0305	305	
0265	0240	240	
0266	0323	323	
0267	0240	240	
0270	0241	241	
0271	0271	.	/ -16 (-14 DEC)
0272	0315	315	/ MEAN RATIO IS
0273	0305	305	
0274	0301	301	
0275	0316	316	
0276	0240	240	
0277	0322	322	
0300	0301	301	
0301	0324	324	
0302	0311	311	
0303	0317	317	
0304	0240	240	
0305	0305	.	/ -13 (-11 DEC)
0306	0323	323	/ STD DEV IS
0307	0324	324	
0310	0304	304	
0311	0240	240	
0312	0304	304	
0313	0305	305	
0314	0326	326	
0315	0240	240	
0316	0311	311	
0317	0323	323	
0320	0240	240	
0321	0321	.	/ -33 (-27 DEC)
0322	0324	324	/ TYPE Y IF DEV SATSFACTORY
0323	0331	311	
0324	0320	320	
0325	0305	305	
0326	0240	240	
0327	0331	311	
0330	0240	240	
0331	0311	311	
0332	0306	306	
0333	0240	240	
0334	0304	304	
0335	0305	305	
0336	0326	326	
0337	0240	240	
0340	0323	323	
0341	0301	301	
0342	0324	324	

0343	0311	311
0344	0323	323
0345	0306	306
0346	0301	301
0347	0303	303
0350	0324	324
0351	0317	317
0352	0322	322
0353	0331	331
0354	0277	277
0355	0240	240
0356	0240	240
0357	0357	•
0360	0255	255
0361	0240	240
0362	0317	317
0363	0316	316
0364	0314	314
0365	0331	311
0366	0240	240
0367	0263	263
0370	0240	240
0371	0323	323
0372	0305	305
0373	0324	324
0374	0323	323

LIST10,

/ -15 (-13 DEC)

/ - ONLY 3 SETS

/ TRIPLE PRECISION ADD OF DOUBLE PRECISION
 / NUMBERS . CALLING SEQUENCE:
 / 1) JMS TRIPAD WITH FIRST ADDRESS IN ACC
 / 2) RETURN SUPERHI ADDRESS IN ACC
 / REQUIRES ' INTEGRATION TIME ' ON PAGE 0 .
 *400

0400	0000	TRIPAD,	0000	
0401	3237		DCA HIPTL	/ FIRST HIGH POINTER
0402	7300		CLL CLA	
0403	3063		DCA OLLO	/ TIDY UP REGISTERS
0404	3062		DCA OLHI	
0405	3061		DCA OLCI	
0406	1030		TAD INTTME	/ HOW MANY ?
0407	7041		CIA	
0410	3236		DCA LOCTME	
0411	1237		TAD HIPTL	
0412	7001		IAC	
0413	3240		DCA LOPTL	
0414	1640	ADDE,	TAD I LOPTL	/ ADD LOW ORDER
0415	1063		TAD OLLO	
0416	3063		DCA OLLO	
0417	7004		RAL	
0420	1637		TAD I HIPTL	/ ADD HIGH ORDER
0421	1062		TAD OLHI	
0422	3062		DCA OLHI	
0423	7004		RAL	/ CARRY
0424	1061		TAD OLCI	
0425	3061		DCA OLCI	
0426	2237		ISZ HIPTL	/ SHIFT ADDRESS BY 2 .
0427	2237		ISZ HIPTL	
0430	2240		ISZ LOPTL	
0431	2240		ISZ LOPTL	
0432	2236		ISZ LOCTME	/ FINISHED ?
0433	5214		JMP ADDE	/ NO
0434	1060		TAD OLCIPT	/ YES
0435	5600		JMP I TRIPAD	
0436	0000	LOCTME,	0000	
0437	0000	HIPTL,	0000	
0440	0000	LOPTL,	0000	

/ DIVIDE TRIPLE PRECISION NUMBER BY A POWER
 / OF TWO . CALLING SEQUENCE:
 / 1) JMS TRIPDV WITH HIGH ADDRESS IN ACC
 / 2) RETURN ACC CLEAR AND RESULT IN THE
 / MEDIUM AND LOW ORDER PART OF T. P. .
 / DIVISOR IN ' INTTME ' ON PAGE 0 .

0441	0000	TRIPDV,	0000	
0442	3313		DCA QUOTHI	/ POINTER

0443	1313		TAD QUOTHI	
0444	7001		IAC	
0445	3314		DCA QUOTMD	
0446	1314		TAD QUOTMD	
0447	7001		IAC	
0450	3315		DCA QUOTLO	
0451	3316		DCA POWER	
0452	7100		CLL	
0453	1030		TAD INITME	/ MUST BE POWER OF 2 !!
0454	7010		RAR	
0455	7010		RAR	
0456	2316		ISZ POWER	/ TWOS EXPONENT
0457	7420		SNL	
0460	5255		JMP .-3	
0461	7200		CLA	/ IF NOT 2**N
0462	1316		TAD POWER	
0463	7041		CIA	
0464	3316		DCA POWER	
0465	7100	TWOSDV,	CLL	
0466	1713		TAD I QUOTHI	/ FETCH PARTS NOW
0467	7010		RAR	
0470	3713		DCA I QUOTHI	
0471	1714		TAD I QUOTMD	
0472	7010		RAR	
0473	3714		DCA I QUOTMD	
0474	1715		TAD I QUOTLO	
0475	7010		RAR	
0476	3715		DCA I QUOTLO	
0477	7004		RAL	
0500	3317		DCA ROUND	/ SAVE ROUND OFF BIT
0501	2316		ISZ POWER	/ FINISHED ?
0502	5265		JMP TWOSDV	/ NO
0503	7300		CLA CLL	/ YES
0504	1715		TAD I QUOTLO	
0505	1317		TAD ROUND	
0506	3715		DCA I QUOTLO	
0507	7004		RAL	/ CARRY
0510	1714		TAD I QUOTMD	
0511	3714		DCA I QUOTMD	
0512	5641		JMP I TRIPDV	
0513	0000	QUOTHI,	0000	
0514	0000	QUOTMD,	0000	
0515	0000	QUOTLO,	0000	
0516	0000	POWER,	0000	
0517	0000	ROUND,	0000	
0520	0000	NWRITE,	0000	/ WRITE OUT MESSAGES .
0521	7100		CLL	/ IN AFTER JMS FROM A
0522	3010		DCA 10	/ TAD CHARXX AS IN *200 .
0523	1720		TAD I NWRITE	/ AFTER JMS IS OCTAL OF
0524	3317		DCA ROUND	/ CHARACTERS AS *200 .

0525	2320		ISZ NWRITE	
0526	6046		TLS	
0527	1410		TAD I 10	
0530	4447		JMS I TYPE	
0531	2317		ISZ ROUND	
0532	5327		JMP .-3	
0533	5720		JMP I NWRITE	
0534	0000	NBELL,	0000	/ RING BELL FOUR TIMES
0535	7300		CLA CLL	
0536	4446		JMS I CRLF	
0537	1346		TAD M4	
0540	3317		DCA ROUND	
0541	1347		TAD K207	
0542	4447		JMS I TYPE	
0543	2317		ISZ ROUND	
0544	5341		JMP .-3	
0545	5734		JMP I NBELL	
0546	7774	M4,	-4	
0547	0207	K207,	207	
0550	0000	NLISN,	0000	/ READ X OR S FROM THE
0551	6031		KSF	/ KEYBOARD
0552	5351		JMP .-1	
0553	6036		KRB	
0554	6046		TLS	
0555	7100		CLL	
0556	3032		DCA XORSOR	
0557	5750		JMP I NLISN	
0560	0000	NEXPON,	0000	/ RAISE ACC TO A
0561	3317		DCA ROUND	/ POWER OF TWO
0562	1717		TAD I ROUND	
0563	7041		CIA	
0564	3316		DCA POWER	
0565	7001		IAC	
0566	7004		RAL	
0567	2316		ISZ POWER	
0570	5366		JMP .-2	
0571	3717		DCA I ROUND	
0572	5760		JMP I NEXPON	

/ SIGNED DECIMAL PRINT , DOUBLE PRECISION
 / CALLING SEQUENCE:
 / 1) JMS SDPRNT / CALLED
 / 2) HIADDR / ADDRESS OF HIGH ORDER
 *600

0600	0000	SDPRNT,	0000	
0601	4550		JMS I ZERDEC	/ ZERO CHECK
0602	1600		TAD I SDPRNT	/ GET ADDRESS
0603	3302		DCA SDGET	
0604	1702		TAD I SDGET	/ HIGH ORDER WORD
0605	7700		SMA CLA	/ NEGATIVE ?
0606	1271		TAD SDPLUS	/ NO
0607	1272		TAD SDMNS	/ YES
0610	1270		TAD SDTWO	
0611	4330		JMS NTYPE	
0612	1702		TAD I SDGET	
0613	7510		SPA	/ POSITIVE ?
0614	7060		CMA CML	
0615	3274		DCA SDHIGH	
0616	2302		ISZ SDGET	
0617	1702		TAD I SDGET	/ LOW ORDER WORD
0620	7430		SZL	/ LINK SET ?
0621	7141		CMA CLL IAC	
0622	7430		SZL	/ OVERFLOW ?
0623	2274		ISZ SDHIGH	
0624	3275		DCA SDLOW	
0625	1266		TAD SDLOOP	/ SET DIGIT COUNTER
0626	3273		DCA SDCNT	
0627	1267		TAD SDADDR	/ SET POINTER
0630	3303		DCA SDPTR	
0631	2200		ISZ SDPRNT	/ SET LINKAGE
0632	1703	SDARND,	TAD I SDPTR	/ POWER OF TEN
0633	2303		ISZ SDPTR	
0634	3276		DCA SDHSUB	
0635	1703		TAD I SDPTR	
0636	2303		ISZ SDPTR	
0637	3277		DCA SDLSUB	
0640	7100	SDDO,	CLL	/ DOUBLE PRECISION
0641	1277		TAD SDLSUB	/ SUBTRACTION
0642	1275		TAD SDLOW	
0643	3301		DCA SDTEML	
0644	7004		RAL	
0645	1276		TAD SDHSUB	
0646	1274		TAD SDHIGH	
0647	7510		SPA	/ UNDERFLOW ?
0650	5256		JMP SDOUT	/ NO
0651	2300		ISZ SDBOX	/ YES
0652	3274		DCA SDHIGH	

0653	1301		TAD SDTEML	
0654	3275		DCA SDLOW	
0655	5240		JMP SDDO	
0656	7200	SDOUT,	CLA	/ PICK UP DIGIT
0657	1300		TAD SDBOX	
0660	1270		TAD SDTWO	
0661	4551		JMS I ZTYPE	
0662	3300		DCA SDBOX	
0663	2273		ISZ SDCNT	/ TYPED 7 DIGITS ?
0664	5232		JMP SDARND	/ NO
0665	5600		JMP I SDPRNT	/ YES
0666	7771	SDLOOP,	-7	/ COUNT 7
0667	0704	SDADDR,	SDCONL	
0670	0260	SDTWO,	260	/ BASE FOR DIGITS
0671	7763	SDPLUS,	-15	
0672	7775	SDMNS,	-3	
0673	0000	SDCNT,	0	/ STORAGE LOCATIONS
0674	0000	SDHIGH,	0	
0675	0000	SDLOW,	0	
0676	0000	SDHSUB,	0	
0677	0000	SDLSUB,	0	
0700	0000	SDBOX,	0	
0701	0000	SDTEML,	0	
0702	0000	SDGET,	0	
0703	0000	SDPTR,	0	
0704	7413	SDCONL,	7413	/ POWERS OF TEN
0705	6700		6700	/ -1,000,000
0706	7747		7747	/ -100,000
0707	4540		4540	
0710	7775		7775	/ -10,000
0711	4360		4360	
0712	7777		7777	/ -1,000
0713	6030		6030	
0714	7777		7777	/ -100
0715	7634		7634	
0716	7777		7777	/ -10
0717	7766		7766	
0720	8777		7777	/ -1
0721	7777		7777	
0722	0000	NCRLF,	0000	/ CARRIAGE RETURN
0723	1336		TAD K215	/ AND LINE FEED
0724	4330		JMS NTYPE	
0725	1337		TAD K212	
0726	4330		JMS NTYPE	
0727	5722		JMP I NCRLF	
0730	0000	NTYPE,	0000	/ TYPE CHARACTER
0731	6041		TSF	/ FROM KEYBOARD

0732	5331		JMP	.-1	
0733	6046		TLS		
0734	7300		CLA	CLL	
0735	5730		JMP	I NTYPE	
0736	0215	K215,		215	
0737	0212	K212,		212	
0740	0000	NTAB,	0000		/ TAB 10 SPACES
0741	1044		TAD	M3	
0742	3350		DCA	TABCT	
0743	1351		TAD	K240	
0744	4330		JMS	NTYPE	
0745	2350		ISZ	TABCT	
0746	5343		JMP	.-3	
0747	5740		JMP	I NTAB	
0750	0000	TABCT,	0000		
0751	0240	K240,		240	
0752	0400	DEPCHG,		3000	
0753	1103		TAD	LOCTON	
0754	3374		DCA	XORS LC	
0755	5752		JMP	I DEPCHG	
0756	0000	DEPXOS,	0000		/ DEPOSIT X OR S
0757	7300		CLA	CLL	/ STARTING 3600
0760	4340		JMS	NTAB	
0761	1032		TAD	XORSOR	
0762	4330		JMS	NTYPE	
0763	4322		JMS	NCRLF	
0764	4322		JMS	NCRLF	
0765	1070		TAD	GMMED	
0766	3774		DCA	I XORS LC	
0767	2374		ISZ	XORS LC	
0770	1071		TAD	GMLOW	
0771	3774		DCA	I XORS LC	
0772	2374		ISZ	XORS LC	
0773	5756		JMP	I DEPXOS	
0774	0000	XORS LC,	0000		

/ READ VOLTMETER VALUES AND FIND THE MEAN
*1000

1000	0000	READVM,	0000	/ CLEAR LOCATIONS
1001	7300		CLA CLL	
1002	1327		TAD MMM6	
1003	3155		DCA TABCNT	
1004	7300		CLA CLL	
1005	3067		DCA GMHI	
1006	3070		DCA GMED	
1007	3071		DCA GMLOW	
1010	1031		TAD GMTIME	
1011	7041		CIA	
1012	3054		DCA GMCT	
1013	7300	MORE,	CLA CLL	/ MORE TO DO !
1014	1064		TAD STORE	
1015	3244		DCA POINTR	
1016	1030		TAD INTIME	
1017	7041		CIA	
1020	3326		DCA MINTT	/ NEGATE
1021	6322		6322	/ SET FLAG FOR A
1022	6301	READVT,	6301	/ VOLTMETER VALUE
1023	5222		JMP .-1	
1024	6302		6302	/ GET HIGH PART
1025	3644		DCA I POINTR	
1026	2244		ISZ POINTR	
1027	6304		6304	/ GET LOW PART
1030	3644		DCA I POINTR	
1031	2244		ISZ POINTR	
1032	6322		6322	/ SET FLAG
1033	2326		ISZ MINTT	/ FINISHED ?
1034	5222		JMP READVT	/ NO
1035	7300		CLA CLL	/ YES
1036	1030		TAD INTIME	
1037	7041		CIA	/ BCD TO BINARY
1040	3326		DCA MINTT	/ RESET COUNTER
1041	1064		TAD STORE	
1042	3244		DCA POINTR	
1043	4456	CONVRT,	JMS I DOUBPT	
1044	0000	POINTR,	0000	/ HIGH BCD NUMBER
1045	3324		DCA TEMPAD	
1046	1724		TAD I TEMPAD	
1047	3644		DCA I POINTR	/ IN OLD BCD
1050	2244		ISZ POINTR	
1051	2324		ISZ TEMPAD	
1052	1724		TAD I TEMPAD	
1053	3644		DCA I POINTR	
1054	2244		ISZ POINTR	/ FINISHED ?
1055	2326		ISZ MINTT	

1056	5243	JMP CONVRT	/ NO
1057	7300	CLA CLL	/ YES
1060	1064	TAD STORE	/ ADDRESS IN ACC
1061	4455	JMS I TRIAPT	/ ADDRESS OF OLXI IN
1062	4457	JMS I TRIDIV	/ ACC ON RETURN
1063	1060	TAD OLXIPT	
1064	7001	IAC	
1065	4554	JMS I LINE	
1066	7300	CLA CLL	
1067	1071	TAD GMLOW	
1070	1063	TAD OLLO	
1071	3071	DCA GMLOW	
1072	7004	RAL	
1073	1062	TAD OLHI	
1074	1070	TAD GMMED	
1075	3070	DCA GMMED	
1076	7004	RAL	
1077	1067	TAD GMHI	
1100	3067	DCA GMHI	
1101	2054	ISZ GMCT	/ COUNTER FOR MEAN
1102	5213	JMP MORE	

/ GET MORE AVERAGES FOR GRAND MEAN

1103	1030	TAD INTTME	/ SET UP FOR MEAN
1104	3325	DCA TEMP	
1105	1031	TAD GMTIME	
1106	3030	DCA INTTME	
1107	1066	TAD GMPT	
1110	4457	JMS I TRIDIV	
1111	1325	TAD TEMP	/ RESTORE INTTME
1112	3030	DCA INTTME	
1113	1066	TAD GMPT	
1114	7001	IAC	
1115	3322	DCA GMPRPT	
1116	4446	JMS I CRLF	
1117	4446	JMS I CRLF	
1120	4453	JMS I TAB	
1121	4465	JMS I DECPRT	
1122	0000	GMPRPT, 0000	
1123	5600	JMP I READVM	
1124	0000	TEMPAD, 0000	
1125	0000	TEMP, 0000	
1126	0000	MINTT, 0000	
1127	7772	MMM6, -6	

1130	0000	TSETIS, 0000	/ WHAT IS THIS
1131	7300	STBACK, CLA CLL	/ SET - X OR S ?
1132	1036	TAD CHAR03	

1133	4433	JMS I WRITE	
1134	7764	-14	
1135	6032	KCC	
1136	4450	JMS I LISN	
1137	1032	TAD XORSOR	
1140	3373	DCA SAMPLE	
1141	7300	CLA CLL	
1142	1373	TAD SAMPLE	
1143	1372	TAD M330	
1144	7440	SZA	
1145	7410	SKP	
1146	5365	JMP ONWARD	/ X
1147	7300	CLA CLL	
1150	1373	TAD SAMPLE	
1151	1371	TAD M323	
1152	7440	SZA	
1153	7410	SKP	
1154	5365	JMP ONWARD	/ S
1155	7300	CLA CLL	
1156	1373	TAD SAMPLE	
1157	1370	TAD M310	
1160	7440	SZA	
1161	7410	SKP	
1162	5774	JMP I PARMPT	/ H (HALT)
1163	4451	JMS I BELL	/ ERROR MADE !!
1164	5331	JMP STBACK	
1165	4570	JMS I KORECT	/ CORRECT SYMBOL
1166	4200	JMS READVM	
1167	5730	JMP I TSETIS	
1170	7470	M310,	-310
1171	7455	M323,	-323
1172	7450	M330,	-330
1173	0000	SAMPLE,	0000
1174	4000	PARMPT,	PARM

/ DOUBLE PRECISION BCD TO BINARY CONVERSION
 / CALLING SEQUENCE:
 / 1) JMS DOUBLE
 / 2) ADDRESS OF HIGH
 / RETURN WITH ADDRESS OF HIGH IN ACC

/ ALSO CONTAINS SINGLE PRECISION BCD TO BINARY
 / CALLING SEQUENCE:
 / 1) C(AC) = 3 BCD CHARACTERS
 / 2) JMS BCDBIN
 / RETURN WITH ANSWER IN C(AC)

*1200

1200	0000	DOUBLE,	0000	
1201	7300		CLA CLL	
1202	1600		TAD I DOUBLE	/ FETCH HIGH ADDRESS
1203	3273		DCA LOW1	
1204	2200		ISZ DOUBLE	
1205	1673		TAD I LOW1	/ FETCH HIGH ORDER
1206	4300		JMS BCDBIN	/ CONVERT IT
1207	3274		DCA HIGH1	/ AND STORE
1210	2273		ISZ LOW1	
1211	1673		TAD I LOW1	/ FETCH LOW ORDER
1212	4300		JMS BCDBIN	/ CONVERT IT
1213	3273		DCA LOW1	/ AND STORE
1214	1274		TAD HIGH1	
1215	7112		CLL RTR	
1216	7012		RTR	
1217	7010		RAR	/ MULTIPLY HIGH ORDER
1220	3300		DCA BCDBIN	/ PART BY 128
1221	1300		TAD BCDBIN	
1222	0332		AND K177	
1223	3275		DCA HIGH	
1224	1300		TAD BCDBIN	
1225	7010		RAR	
1226	0330		AND K7600	
1227	3276		DCA LOW	
1230	1274		TAD HIGH1	/ MULTIPLY HIGH ORDER
1231	7104		CLL RAL	/ BY THREE
1232	1274		TAD HIGH1	/ FORM 128*HIGH-3*HIGH
1233	7141		CIA CLL	
1234	1276		TAD LOW	
1235	3276		DCA LOW	
1236	7420		SNL	
1237	7040		CMA	
1240	1275		TAD HIGH	
1241	3275		DCA HIGH	/ 125*HIGH
1242	1275		TAD HIGH	/ NOW MULTIPLY BY 8

1243	7106		CLL RTL	
1244	7004		RAL	
1245	0331		AND K7770	/ MASK 9 BITS
1246	3275		DCA HIGH	
1247	1276		TAD LOW	
1250	7106		CLL RTL	
1251	7004		RAL	
1252	3276		DCA LOW	
1253	1276		TAD LOW	
1254	7004		RAL	
1255	0327		AND K7	/ 3 BITS
1256	1275		TAD HIGH	
1257	3275		DCA HIGH	
1260	1276		TAD LOW	
1261	0331		AND K770	/ 9 BITS
1262	7100		CLL	
1263	1273		TAD LOW1	/ ADD LOW PART
1264	3276		DCA LOW	/ AND STORE
1265	1275		TAD HIGH	
1266	7430		SZL	
1267	7001		IAC	
1270	3275		DCA HIGH	
1271	1277		TAD HIPTX	/ RETURN WITH HIGH
1272	5600		JMP I DOUBLE	/ ADRESS IN ACC
1273	0000	LOW1,	0	
1274	0000	HIGH1,	0	
1275	0000	HIGH,	0	
1276	0000	LOW,	0	
1277	1275	HIPTX,	HIGH	

/ SINGLE PRECISION CONVERSION

1300	0000	BCDBIN,	0000	
1301	3275		DCA HIGH	
1302	1275		TAD HIGH	
1303	0333		AND K7400	/ LEFT DIGIT
1304	7112		CLL RTR	
1305	3276		DCA LOW	
1306	1276		TAD LOW	
1307	7010		RAR	
1310	1276		TAD LOW	
1311	7041		CIA	
1312	1275		TAD HIGH	
1313	3275		DCA HIGH	
1314	1275		TAD HIGH	
1315	0326		AND K7760	
1316	7112		CLL RTR	
1317	3276		DCA LOW	
1320	1276		TAD LOW	
1321	7010		RAR	

1322	1276		TAD LOW
1323	7041		CIA
1324	1275		TAD HIGH
1325	5700		JMP I BCDBIN

1326	7760	K7760,	7760
1327	0007	K7,	7
1330	7600	K7600,	7600
1331	7770	K7770,	7770
1332	0177	K177,	177
1333	7400	K7400,	7400

1334	0000	STARS,	0000	/ LINE OF STARS AND
1335	4446		JMS I CRLF	/ CRLF'S TO END THE
1336	1347		TAD M70	/ PROGRAM
1337	3350		DCA STARLN	
1340	1351		TAD KK252	
1341	4447		JMS I TYPE	
1342	2350		ISZ STARLN	
1343	5340		JMP .-3	
1344	4446		JMS I CRLF	
1345	5353		JMP .+6	
1346	5752		JMP I PARMPT	
1347	7672	M70,	-70	
1350	0000	STARLN,	0000	
1351	0252	KK252,	252	
1352	3532	PARMPT,	PARM	

1353	4446		JMS I CRLF
1354	4446		JMS I CRLF
1355	4446		JMS I CRLF
1356	4446		JMS I CRLF
1357	4446		JMS I CRLF
1360	4446		JMS I CRLF
1361	4446		JMS I CRLF
1362	4446		JMS I CRLF
1363	4446		JMS I CRLF
1364	4446		JMS I CRLF
1365	4446		JMS I CRLF
1366	3077		DCA TEMTQ
1367	1376		TAD MN260
1370	3777		DCA I LCN1
1371	1375		TAD MIN3
1372	3774		DCA I LCN1
1373	5346		JMP PAROUT
1374	3372	LCN1,	3372
1375	7775	MIN3,	-3
1376	7520	MN260,	-260
1377	3346	LCN1,	3346

/ DOUBLE PRECISION DIVIDE
*1400

1400	0000	DVDRAT,	0000	/ EXPECT STORED
1401	1117		TAD DVDPT	/ DV1, DV2, DVSOR1,
1402	3206		DCA MULT+1	/ AND DVSOR2 .
1403	1123		TAD TEN5PT	
1404	3207		DCA MULT+2	
1405	4521	MULT,	JMS I DMULPT	
1406	0000		0000	
1407	0000		0000	
1410	3113		DCA DV1	
1411	1132		TAD DV2PT	
1412	3126		DCA ADD	
1413	1526		TAD I ADD	
1414	3114		DCA DV2	
1415	2126		ISZ ADD	
1416	1526		TAD I ADD	
1417	3115		DCA DV3	
1420	2126		ISZ ADD	
1421	1526		TAD I ADD	
1422	3116		DCA DV4	
1423	1120		TAD DVSPT	
1424	3227		DCA ADDRS1	
1425	1117		TAD DVDPT	
1426	4522		JMS I DBDVPT	
1427	0000	ADDRS1,	0000	
1430	3126		DCA ADD	
1431	1526		TAD I ADD	
1432	3107		DCA QUOT1	
1433	2126		ISZ ADD	
1434	1526		TAD I ADD	
1435	3110		DCA QUOT2	
1436	1127		TAD QUOTPT	
1437	4554		JMS I LINE	
1440	5600		JMP I DVDRAT	
1441	0000	HAPPYS,	0000	/ 'Y' TYPED IF THE
1442	1042		TAD CHAR07	/ RESULTS ARE SAT-
1443	4433		JMS I WRITE	/ ISFACTORY .THREE
1444	7745		-33	/ MORE SETS OTHER-
1445	4453		JMS I TAB	/ WISE .
1446	4453		JMS I TAB	
1447	6032		KCC	/ 'S' STARTS !!
1450	4450		JMS I LISN	
1451	7000		NOP	
1452	4575		JMS I MUMCRT	
1453	4574		JMS I DELETE	

1454	7000		NOP	
1455	4446		JMS I CRLF	
1456	4446		JMS I CRLF	
1457	4446		JMS I CRLF	
1460	4536		JMS I MEANNF	
1461	0000	SORTNO,	0000	/ DOUBLE PRECISION
1462	7300		CLA CLL	/ SQUARE ROOT OF A
1463	3350		DCA ROOT	/ DOUBLE PRECISION
1464	1352		TAD M1	/ NUMBER , EXPECTED
1465	3351		DCA SQXT	/ IN QUOT1 AND QUOT2
1466	1352		TAD M1	/ WITH RESULT IN
1467	3354		DCA MM	/ QUOT2 .
1470	1110	SQX,	TAD QUOT2	/ SINGLE PRECISION .
1471	1361		TAD SQXT	
1472	3110		DCA QUOT2	
1473	7004		RAL	
1474	1107		TAD QUOT1	
1475	1354		TAD MM	
1476	3107		DCA QUOT1	
1477	7420		SNL	
1500	5331		JMP SQRF	
1501	7100		CLL	
1502	2350		ISZ ROOT	
1503	1351		TAD SQXT	
1504	1356		TAD K4000	
1505	7450		SNA	
1506	5315		JMP COMPLX	
1507	7300		CLA CLL	
1510	1353		TAD M2	
1511	1351		TAD SQXT	
1512	3351		DCA SQXT	
1513	7100		CLL	
1514	5270		JMP SQX	
1515	7300	COMPLX,	CLA CLL	/ DOUBLE PRECISION
1516	1353		TAD M2	
1517	1354		TAD MM	
1520	3354		DCA MM	
1521	1354		TAD MM	
1522	1355		TAD K3776	
1523	7450		SNA	
1524	5331		JMP SQRF	
1525	7300		CLA CLL	
1526	1352		TAD M1	
1527	3351		DCA SQXT	
1530	5270		JMP SQX	
1531	7300	SQRF,	CLA CLL	/ ROUND OFF
1532	1351		TAD SQXT	
1533	7041		CIA	
1534	1110		TAD QUOT2	

1535	3110		DCA QUOT2
1536	1110		TAD QUOT2
1537	1110		TAD QUOT2
1540	1351		TAD SQXT
1541	7500		SMA
1542	2350		ISZ ROOT
1543	7300		CLA CLL
1544	1350		TAD ROOT
1545	3110		DCA QUOT2
1546	3107		DCA QUOT1
1547	5661		JMP I SQRTNO
1550	0000	ROOT,	0000
1551	0000	SQXT,	0000
1552	7777	M1,	-1
1553	7776	M2,	-2
1554	0000	MM,	0000
1555	3776	K3776,	3776
1556	4000	K4000,	4000

		*1600		
1600	0000	RATIO,	0000	/ EVALUATION OF AN
1601	3164		DCA NEWXOS	/ X/S RATIO
1602	1274		TAD M6	
1603	3106		DCA CNTR	
1604	1274		TAD M6	
1605	3155		DCA TABCNT	
1606	1103		TAD LOCTON	
1607	3275		DCA XORSL	
1610	5217		JMP RATCON	
1611	0000	AGNRAT,	0000	/ SET COUNTERS
1612	7300		CLA CLL	
1613	1044		TAD M3	
1614	3106		DCA CNTR	
1615	1274		TAD M6	
1616	3155		DCA TABCNT	
1617	3272	RATCON,	DCA SOXCNT	
1620	7300		CLA CLL	
1621	1272	RATIOT,	TAD SOXCNT	
1622	1106		TAD CNTR	
1623	7650		SNA CLA	
1624	5264		JMP ISWHAT	/ X/S OR S/X ?
1625	1275		TAD XORSL	
1626	4547		JMS I STXORS	
1627	7300		CLA CLL	
1630	1273		TAD XOSCNT	
1631	7440		SZA	
1632	5235		JMP .+3	
1633	2273		ISZ XOSCNT	
1634	5253		JMP FINELY	
1635	7300		CLA CLL	
1636	1112	SETX,	TAD DVSOR2	/ AN X/S !
1637	3072		DCA TEMPTM	
1640	1114		TAD DV2	
1641	3112		DCA DVSOR2	
1642	1072		TAD TEMPTM	
1643	3114		DCA DV2	
1644	1111		TAD DVSOR1	
1645	3072		DCA TEMPTM	
1646	1113		TAD DV1	
1647	3111		DCA DVSOR1	
1650	1072		TAD TEMPTM	
1651	3113		DCA DV1	
1652	3273		DCA XOSCNT	
1653	4537	FINELY,	JMS I DVDRTO	/ DIVIDE IT
1654	1107		TAD QUOY1	
1655	3675		DCA I XORSL	
1656	2275		ISZ XORSL	
1657	1110		TAD QUOT2	

1660	3675		DCA I XORSL	
1661	2272		ISZ SOXCNT	
1662	2275		ISZ XORSL	
1663	5221		JMP RATIO	
1664	1106	ISWHAT,	TAD CNTR	/ RETURN WHERE ?
1665	7041		CIA	
1666	1274		TAD M6	
1667	7440		SZA	
1670	5611		JMP I AGNRAT	/ 3 MORE SETS
1671	5600		JMP I RATIO	/ MAIN SIX
1672	0000	SOXCNT,	0000	
1673	0000	XOSCNT,	0000	
1674	7772	M6,	-6	
1675	0000	XORSL,	0000	
1676	0000	STDDVN,	0000	/ EVALUATE STANDARD
1677	7300		CLA CLL	/ DEVIATION FOR
1700	1103		TAD LOCTON	/ FIRST SIX VALUES
1701	3375		DCA XORSCB	
1702	3105		DCA NUMT	
1703	3376		DCA STDCNT	
1704	1274		TAD M6	
1705	3106		DCA CNTR	
1706	5312		JMP STDON	
1707	0000	STDTWO,	0000	/ AFTER 3 MORE SETS
1710	7300		CLA CLL	
1711	3376		DCA STDCNT	
1712	7300	STDON,	CLA CLL	/ SUM OF SQUARES
1713	1376		TAD STDCNT	
1714	1106		TAD CNTR	
1715	7700		SMA CLA	
1716	5366		JMP STDFOR	
1717	1375		TAD XORSCB	
1720	3377		DCA XORS	
1721	2377		ISZ XORS	
1722	1775		TAD I XORSCB	
1723	7040		CMA	
1724	3113		DCA DV1	
1725	1777		TAD I XORS	
1726	7450		SNA	
1727	5333		JMP .+4	
1730	7041		CIA	
1731	3114		DCA DV2	
1732	5335		JMP .+3	
1733	2113		ISZ DV1	
1734	3114		DCA DV2	
1735	1114		TAD DV2	
1736	1110		TAD QUOT2	
1737	3114		DCA DV2	
1740	7004		RAL	

1741	1113		TAD DV1	
1742	1107		TAD QUOT1	
1743	3113		DCA DV1	
1744	7100		CLL	
1745	1117		TAD DVDPT	
1746	3352		DCA STDML+1	
1747	1117		TAD DVDPT	
1750	3353		DCA STDML+2	
1751	4521	STDML,	JMS I DMULPT	/ SQUARE
1752	0000		0000	
1753	0000		0000	
1754	7640		SZA CLA	
1755	4553		JMS I CNTRO	
1756	1532		TAD I DV2PT	
1757	7640		SZA CLA	
1760	4553		JMS I CNTRO	
1761	4565		JMS I DEVSET	
1762	2375		ISZ XORSCB	
1763	2375		ISZ XORSCB	
1764	2376		ISZ STDCNT	
1765	5312		JMP STDON	/ FINISHED ?
1766	1106	STDFOR,	TAD CNTR	
1767	7041		CIA	
1770	1274		TAD M6	
1771	7650		SNA CLA	
1772	5676		JMP I STDDVN	/ YES
1773	4557		JMS I NEG1	/ NO
1774	5707		JMP I STD1WO	
1775	0000	XORSCB,	0000	
1776	0000	STDCNT,	0000	
1777	0000	XORS,	0000	

/ SIGNED DOUBLE PRECISION MULTIPLY
 / CALLING SEQUENCE:
 / 1) JMS DMUL
 / 2) ADDRESS OF MULTIPLICAND (HIGH)
 / ADDRESS OF MULTIPLIER (HIGH)
 / RETURN , HIGH PRODUCT IN AC , NEXT IN
 / B , C , AND D , ETC .
 *2000

2000	0000	DMUL,	0000	
2001	7300		CLA CLL	
2002	4306		JMS TSIGN	/ FETCH AND SET SIGN
2003	1337		TAD MLTH	
2006	3334		DCA MULTH	/ HIGH MULTIPLICAND
2007	1336		TAD MLTL	
2010	3335		DCA MULTL	/ LOW MULTIPLICAND
2011	4306		JMS TSIGN	/ FETCH AND SET SIGN
2012	1335		TAD MULTL	
2013	3301		DCA MP2	
2014	1336		TAD MLTL	
2015	4344		JMS MP4	/ MULTIPLY LOWS
2016	3343		DCA D	
2017	1373		TAD MP5	
2020	3342		DCA C	
2021	1334		TAD MULTH	
2022	3301		DCA MP2	
2023	1336		TAD MLTL	
2024	4344		JMS MP4	/ MULTIPLY HIGHS
2025	1342		TAD C	
2026	3342		DCA C	
2027	7004		RAL	/ GET CARRY
2030	1373		TAD MP5	
2031	3341		DCA B	
2032	7004		RAL	
2033	3340		DCA A	
2034	1335		TAD MULTL	
2035	3301		DCA MP2	
2036	1337		TAD MLTH	
2037	4344		JMS MP4	
2040	1342		TAD C	
2041	3342		DCA C	
2042	7004		RAL	
2043	1373		TAD MP5	
2044	1341		TAD B	
2045	3341		DCA B	
2046	7004		RAL	
2047	1340		TAD A	
2050	3340		DCA A	
2051	1334		TAD MULTH	

2052	3301	DCA MP2	
2053	1337	TAD MLTH	
2054	4344	JMS MP4	
2055	1341	TAD B	
2056	3341	DCA B	
2057	7004	RAL	
2060	1373	TAD MP5	
2061	1340	TAD A	
2062	5600	JMP I DMUL	/ EXIT WITH HIGH
2063	7402	HLT	

		MP1,		
2106	0000	TSIGN,	0000	
2107	1600		TAD I DMUL	/ FETCH ADDRESS
2110	3340		DCA ADDRSS	
2111	1740		TAD I ADDRSS	/ HIGH ORDER
2112	7100		CLL	
2113	7510		SPA	/ IS IT < 0 ?
2114	7060		CMA CML	/ YES
2115	3337		DCA MLTH	
2116	2340		ISZ ADDRSS	
2117	1740		TAD I ADDRSS	/ LOW ORDER
2120	7430		SZL	
2121	7141		CMA CLL IAC	
2122	3336		DCA MLTL	
2126	7430		SZL	
2127	2337		ISZ MLYH	
2130	2200		ISZ DMUL	
2131	5706		JMP I TSIGN	

2134	0000	MULTH,	0000	
2135	0000	MULTL,	0000	
2136	0000	MLTL,	0000	
2137	0000	MLTH,	0000	
		ADDRSS,		
2140	0000	A,	0	
2141	0000	B,	0	
2142	0000	C,	0	
2143	0000	D,	0	
2144	0000	MP4,	0000	/ UNSIGNED MULTIPLY
2145	3306		DCA MP1	
2146	3373		DCA MP5	
2147	1374		TAD M12	/ 12 BITS
2150	3372		DCA MP3	
2151	7100		CLL	
2152	1306		TAD MP1	
2153	7010		RAR	
2154	3306		DCA MP1	
2155	1373		TAD MP5	

2156	7420		SNL	/ A 1 ?
2157	5362		JMP .+3	/ NO
2160	7100		CLL	/ YES
2161	1301		TAD MP2	
2162	7010		RAR	
2163	3373		DCA MP5	
2164	2372		ISZ MP3	/ 12 BITS ?
2165	5352		JMP MP4+6	/ NO
2166	1306		TAD MP1	/ YES
2167	7010		RAR	
2170	7100		CLL	
2171	5744		JMP I MP4	
2172	0000	MP3,	0	
2173	0000	MP5,	0	
2174	7764	M12,	-14	


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/ DOUBLE PRECISION DIVIDE
/ CALLING SEQUENCE:
/   1) C(AC)=ADDRESS HIGH ORDER DIVIDEND
/   2) JMS DUBDIV
/   3) ADDRESS OF HIGH ORDER DIVISOR
/ RETURN : C(AC) =HIGH QUOTIENT
/           C(DIVND4)=LOW QUOTIENT
/           C(DIVND1)=HIGH REMAINDER
/           C(DIVND2)=LOW REMAINDER
/ EXIT WITH ADDRESS

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*2200
2200 0000 DUBDIV, 0000 / DIVIDEND ADDRESS
2201 3334 DCA ADDR5
2202 1734 TAD I ADDR5 / HIGH DIVIDEND
2205 3335 DCA DIVND1
2206 2334 ISZ ADDR5
2207 1734 TAD I ADDR5 / DIVIDEND
2210 3336 DCA DIVND2
2211 2334 ISZ ADDR5
2212 1734 TAD I ADDR5 / DIVIDEND
2213 3337 DCA DIVND3
2214 2334 ISZ ADDR5
2215 1734 TAD I ADDR5 / DIVIDEND
2216 3340 DCA DIVND4
2217 5237 JMP DIVG01 / GET DIVISOR
2220 1342 RDCONT, TAD LDIVSR
2221 1336 TAD DIVND2
2222 3336 DCA DIVND2
2223 7004 RAL
2224 1341 TAD HDIVSR
2225 1335 TAD DIVND1
2226 3335 DCA DIVND1
2227 7100 CLL
2230 1335 TAD DIVND1 / HIGH=0 ?
2231 7440 SZA
2232 5234 JMP .+2 / NO
2233 1336 TAD DIVND2 / YES
2234 7510 SPA
2235 5347 JMP RDCON+1
2236 5346 JMP RDCON

/ FETCH DIVISOR
2237 1600 DIVG01, TAD I DUBDIV
2240 2200 ISZ DUBDIV
2241 3334 DCA ADDR5
2242 1734 TAD I ADDR5 / HIGH DIVISOR

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2243	7100		CLL	
2244	7500		SMA	/ DIVISOR > 0 ?
2245	7060		CMA CML	/ YES
2246	3341		DCA HDIVSR	
2247	2334		ISZ ADDRS	
2250	1734		TAD I ADDRS	/ LOW DIVISOR
2251	7430		SZL	
2255	7141		CMA IAC CLL	
2256	3342		DCA LDIVSR	
2257	7430		SZL	/ CARRY ?
2260	2341		ISZ HDIVSR	/ YES
2261	1345		TAD M25	
2262	3344		DCA DIVCNT	
2263	7100		CLL	
2264	5307		JMP DIV2	
2265	1336	DIV3,	TAD DIVND2	/ SHIFT HIGH
2266	7004		RAL	/ DIVIDEND LEFT
2267	3336		DCA DIVND2	
2270	1335		TAD DIVND1	
2271	7004		RAL	
2272	3335		DCA DIVND1	
2273	1336		TAD DIVND2	/ COMPARE DIVISOR
2274	1342		TAD LDIVSR	/ WITH DIVISOR
2275	3334		DCA ADDRS	
2276	7004		RAL	/ CARRY
2277	1335		TAD DIVND1	
2300	1341		TAD HDIVSR	
2301	7420		SNL	
2302	5306		JMP DIV2-1	
2303	3335		DCA DIVND1	
2304	1334		TAD SDDRS	
2305	3336		DCA DIVND2	
2306	7200		CLA	
2307	1340	DIV2,	TAD DIVND4	/ ROTATE LOW
2310	7004		RAL	
2311	3340		DCA DIVND4	
2312	1337		TAD DIVND3	/ QUOTIENT BITS
2313	7004		RAL	
2314	3337		DCA DIVND3	
2315	2344		ISZ DIVCNT	/ DONE 24 ?
2316	5265		JMP DIV3	/ NO
2317	7300		CLA CLL	/ YES
2320	1336		TAD DIVND2	
2321	1336		TAD DIVND2	
2322	3336		DCA DIVND2	
2323	7004		RAL	
2324	1335		TAD DIVND1	
2325	1335		TAD DIVND1	
2326	3335		DCA DIVND1	
2327	5220		JMP RDCONT	

2331	1333	OUT,	TAD DVNDPT	/ EXIT
2332	5600		JMP I DUBDIV	
2333	2337	DVNDPT,	DIVND3	
2334	0000	ADDRS,	0	
2335	0000	DIVND1,	0	
2336	0000	DIVND2,	0	
2337	0000	DIVND3,	0	
2340	0000	DIVND4,	0	
2341	0000	HDIVSR,	0	
2342	0000	LDIVSR,	0	
2344	0000	DIVCNT,	0	
2345	7747	M25,	-31	/ -25 (10)
2346	2340	RDCON,	ISZ DIVND4	
2347	7300		CLA CLL	
2350	5331		JMP OUT	
2355	3371		DCA SET1	/ SORT SETS
2356	1771		TAD I SET1	
2357	7650		SNA CLA	
2360	5364		JMP .+4	
2361	1371		TAD SET1	
2362	3772		DCA I SET2	
2363	5773		JMP I SET3	
2364	2371		ISZ SET1	
2365	1771		TAD I SET1	
2366	7640		SZA CLA	
2367	5363		JMP .-4	
2370	5375		JMP .+5	
2371	0000	SET1,	0000	
2372	3156	SET2,	3156	
2373	3076	SET3,	3076	
2374	3140	SET4,	3140	
2375	3561		DCA I R2CNT	
2376	5774		JMP I SET4	

/ EVALUATION OF STANDARD DEVIATION
 *2400

2400	0000	TWCASE,	0000	/ SET OF SIX
2401	1105		TAD NUMT	
2402	7640		SZA CLA	
2403	5274		JMP NFF5	
2404	5271		JMP FIF5	
2405	4577	TWCASS,	JMS I NUMTCR	/ SET OF THREE
2406	1104		TAD LOCTEN	
2407	3306		DCA XOSSTD	
2410	3336		DCA AGNCNT	
2411	3115		DCA DV3	
2412	3116		DCA DV4	
2413	1336	CASEON,	TAD AGNCNT	/ SET UP ARRAY
2414	1106		TAD CNTR	
2415	7700		SMA CLA	
2416	5237		JMP DVCASE	
2417	1306		TAD XOSSTD	
2420	3305		DCA STDXOS	
2421	2305		ISZ STDXOS	
2422	1116		TAD DV4	
2423	1705		TAD I STDXOS	
2424	3116		DCA DV4	
2425	7004		RAL	
2426	1115		TAD DV3	
2427	1706		TAD I XOSSTD	
2430	3115		DCA DV3	
2431	7430		SZL	
2432	4553		JMS I CNTRO	
2433	2306		ISZ XOSSTD	
2434	2306		ISZ XOSSTD	
2435	2336		ISZ AGNCNT	/ ALL DONE ?
2436	5213		JMP CASEON	/ NO
2437	1105	DVCASE,	TAD NUMT	/ SO ADD UP
2440	3112		DCA DVSOR2	
2441	3111		DCA DVSOR1	
2442	3113		DCA DV1	
2443	3114		DCA DV2	
2444	1120		TAD DVSPT	
2445	3250		DCA ADDRS5	
2446	1117		TAD DVDPT	
2447	4522		JMS I DBDVPT	
2450	0000	ADDRS5,	0000	/ FIND DEVIATION
2451	3307		DCA RESULT	
2452	1707		TAD I RESULT	
2453	3107		DCA QUOT1	
2454	2307		ISZ RESULT	
2455	1707		TAD I RESULT	

2456	3110		DCA QUOT2	
2457	4544		JMS I SQRT	/ TAKE ROOT
2460	1041		TAD CHAR06	
2461	4433		JMS I WRITE	
2462	7765		-13	
2463	1127		TAD QUOTPT	
2464	3266		DCA ADDRS4	
2465	4465		JMS I DECPRT	/ PRINT IT
2466	0000	ADDRS4,	0000	
2467	4446		JMS I CRLF	
2470	5600		JMP I TWCASE	
2471	1304	FIRF5,	TAD K5	/ SET OF SIX
2472	3105		DCA NUMT	
2473	5205		JMP TWCASS	
2474	1105	NFF5,	TAD NUMT	/ SET OF THREE
2475	1045		TAD K3	
2476	3105		DCA NUMT	
2477	5205		JMP TWCASS	
2504	0005	K5,	5	
2505	0000	STDXOS,	0	
2506	0000	XOSSTD,	0	
2507	0000	RESULT,	0	
2510	0000		0	
2511	0000	AGAIN3,	0000	/ DEPOSIT AND DO
2512	7300		CLA CLL	/ MORE RATIOS
2513	3336		DCA AGNCNT	
2514	4562		JMS I XOSMB	
2515	1037		TAD CHAR04	
2516	4433		JMS I WRITE	/ DO MESSAGE
2517	7753		-25	
2520	1043		TAD CHAR10	
2521	4433		JMS I WRITE	
2522	7763		-15	
2523	4446		JMS I CRLF	
2524	1336		TAD AGNCNT	
2525	1044		TAD M3	
2526	7700		SMA CLA	
2527	5334		JMP .+5	
2530	2336		ISZ AGNCNT	/ MORE DATA ?
2531	4533		JMS I SETIS	
2532	4535		JMS I DEPOST	
2533	5324		JMP .-7	
2534	4531		JMS I RATAGN	
2535	5711		JMP I AGAIN3	
2536	0000	AGNCNT,	0000	
2537	0000	STXOS,	0000	/ STORE RATIO
2540	3375		DCA XOS	
2541	1775		TAD I XOS	

2542	3111	DCA DVSOR1
2543	2375	ISZ XOS
2544	1775	TAD I XOS
2545	3112	DCA DVSOR2
2546	2375	ISZ XOS
2547	1775	TAD I XOS
2550	1775	TAD I XOS
2551	3113	DCA DV1
2552	2375	ISZ XOS
2553	1775	TAD I XOS
2554	1775	TAD I XOS
2555	3114	DCA DV2
2556	7004	RAL
2557	1113	TAD DV1
2560	3113	DCA DV1
2561	2375	ISZ XOS
2562	1775	TAD I XOS
2563	1111	TAD DVSOR1
2564	3111	DCA DVSOR1
2565	2375	ISZ XOS
2566	1775	TAD I XOS
2567	1112	TAD DVSOR2
2570	3112	DCA DVSOR2
2571	7004	RAL
2572	1111	TAD DVSOR1
2573	3111	DCA DVSOR1
2574	5737	JMP I STXOS
2575	0000	0000

XOS,

/ READY FOR MEAN

/ OVERFLOW PROTECTION

*2600

2600	0000	CNTROS,	0000	/ SEND MESSAGE
2601	7300		CLA CLL	
2602	1206		TAD ERRMES	
2603	4433		JMS I WRITE	
2604	7763		-15	
2605	5600		JMP I CNTROS	
2606	2606	ERRMES,	.	/ -15 (-13 DEC)
2607	0215		215	/ OVERFLOW
2610	0212		212	
2611	0240		240	
2612	0317		317	
2613	0326		326	
2614	0305		305	
2615	0322		322	
2616	0306		306	
2617	0314		314	
2620	0317		317	
2621	0327		327	
2622	0212		212	
2623	0215		215	

2624	0000	DECZER,	0000	/ DECIMAL LOCATION
2625	7300		CLA CLL	
2626	3231		DCA ZRCNT	
2627	3232		DCA ZERCNT	
2630	5624		JMP I DECZER	
2631	0000	ZRCNT,	0	
2632	0000	ZERCNT,	0	

2633	0000	ZRTYPE,	0000	
2634	3264		DCA ZLCN	
2635	1232		TAD ZERCNT	
2636	7640		SZA CLA	
2637	5244		JMP NOZER	
2640	1264		TAD ZLCN	
2641	1266		TAD MZ260	
2642	7650		SNA CLA	
2643	5247		JMP ZERO	
2644	2232	NOZER,	ISZ ZERCNT	/ SUPRESS ZEROS
2645	1264		TAD ZLCN	
2646	5251		JMP .+3	
2647	6046	ZERO,	TLS	/ TYPE A BLANK
2650	1267		TAD B240	
2651	4447		JMS I TYPE	

2652	2231		ISZ ZRCNT
2653	1231		TAD ZRCNT
2654	1265		TAD MMM2
2655	7650		SNA CLA
2656	5260		JMP DECZR
2657	5633		JMP I ZRTYPE

2660	1270	DECZR,	TAD D256
2661	4447		JMS I TYPE
2662	2232		ISZ ZERCNT
2663	5633		JMP I ZRTYPE
2664	0000	ZLCN,	0
2665	7776	MMM2,	-2
2666	0260	MZ260,	260
2667	0240	B240,	240
2670	0256	D256,	256

2671	0000	ONEDV,	0000	/ DIVIDE
2672	1120		TAD DVSPT	
2673	3276		DCA ADDR57	
2674	1117		TAD DVDPT	
2675	4522		JMS I DBDVPT	
2676	0000	ADDR57,	0000	
2677	3310		DCA RESULT	
2700	1710		TAD I RESULT	
2701	3107		DCA QUOT1	
2702	2310		ISZ RESULT	
2703	1710		TAD I RESULT	
2704	3110		DCA QUOT2	
2705	4446		JMS I CRLF	
2706	4446		JMS I CRLF	
2707	5671		JMP I ONEDV	
2710	0000	RESULT,	0000	

2711	0000	LINES,	0000	/ PRINT OUTPUT ON
2712	3325		DCA LINLCN	/ A LINE OF SIX
2713	1155		TAD TABCNT	
2714	7640		SZA CLA	
2715	5322		JMP .+5	
2716	4446		JMS I CRLF	
2717	1330		TAD LCN	
2720	3155		DCA TABCNT	
2721	5324		JMP .+3	
2722	2155		ISZ TABCNT	
2723	7000		NOP	
2724	4465		JMS I DECPRT	/ PRINT ONE
2725	0000	LINLCN,	0000	
2726	4453		JMS I TAB	
2727	5711		JMP I LINES	

2731	0000	MBXOS,	0000	/ X OR S FOR
2732	1164		TAD NEWXOS	/ NEW SET ?
2733	7640		SZA CLA	
2734	5341		JMP .+5	
2735	1345		TAD PL323	
2736	3563		DCA I SAYLCN	
2737	2164		ISZ NEWXOS	
2740	5344		JMP .+4	
2741	1346		TAD PL330	
2742	3563		DCA I SAYLCN	
2743	3164		DCA NEWXOS	
2744	5731		JMP I MBXOS	
2745	0323	PL323,	323	
2746	0330	PL330,	330	

2747	0000	CORECT,	0000	/ OPERATOR CORRECT ?
2750	7300		CLA CLL	
2751	1102		TAD TEMTT	
2752	7640		SZA CLA	
2753	5363		JMP .+8	
2754	1032		TAD XORSOR	
2755	1374		TAD MC323	
2756	7640		SZA CLA	
2757	5372		JMP .+11	
2760	2102		ISZ TEMTT	
2761	4446		JMS I CRLF	
2762	5747		JMP I CORECT	/ YES
2763	1032		TAD XORSOR	
2764	1375		TAD MC330	
2765	7640		SZA CLA	
2766	5372		JMP .+4	
2767	3102		DCA TEMTT	
2770	4446		JMS I CRLF	
2771	5747		JMP I CORECT	/ YES
2772	4446		JMS I CRLF	
2773	5571		JMP I BACKST	/ NO
2774	7455	MC323,	-323	
2775	7450	MC330,	-330	

		*3000		
3000	0000	SETDEV,	0000	/ DEPOSIT DEVIATIONS
3001	7300		CLA CIL	/ SEPARATE FROM THE
3002	1214		TAD DEVT1	/ RATIOS ON *3600
3003	3215		DCA DEVT2	
3004	2215		ISZ DEVT2	
3005	1560		TAD I RICNT	
3006	3614		DCA I DEVT1	
3007	1561		TAD I R2CNT	
3010	3615		DCA I DEVT2	
3011	2214		ISZ DEVT1	
3012	2214		ISZ DEVT1	
3013	5600		JMP I SETDEV	
3014	0000	DEVT1,	0	
3015	0000	DEVT2,	0	
3016	0000	ONGO,	0000	/ INTIME AND GMTIME
3017	1034		TAD CHAR01	/ SETS . HOW MANY ?
3020	4433		JMS I WRITE	
3021	7766		-12	
3022	6032		KCC	
3023	4450		JMS I LISN	
3024	1032		TAD XORSOR	
3025	4360		JMS TEST	/ CORRECT NUMBER ?
3026	3030		DCA INTIME	
3027	4446		JMS I CRLF	
3030	1247		TAD BASE1	
3031	4452		JMS I EXPON	/ EXPONENTIATE
3032	1035		TAD CHAR02	/ INTIME FIRST
3033	4433		JMS I WRITE	
3034	7766		-12	
3035	6032		KCC	
3036	4450		JMS I LISN	
3037	1032		TAD XORSOR	
3040	4360		JMS TEST	/ CORRECT NUMBER ?
3041	3031		DCA GMTIME	
3042	4446		JMS I CRLF	
3043	1250		TAD BASE2	
3044	4452		JMS I EXPON	/ EXPONENTIATE
3045	5616		JMP I ONGO	/ GMTIME NOW
3046	7520	M260,	-260	
3047	0030	BASE1,	INTIME	
3050	0031	BASE2,	GMTIME	
3051	0000	NEGIT,	0000	/ EVALUATE THE NEW
3052	3354		DCA EXTICNT	/ STANDARD DEVIATION

3053	1103	TAD LOCTON	/ SINCE THREE MORE
3054	3355	DCA XOSEXI	/ SETS
3055	1104	TAD LOCTEN	
3056	3357	DCA EXISOX	
3057	1167	TAD EXIRA	
3060	7440	SZA	/ MORE ?
3061	5265	JMP .+4	/ NO
3062	1353	TAD MD9	
3063	3167	DCA EXTRA	
3064	5267	JMP .+3	
3065	1044	TAD M3	
3066	3167	DCA EXTRA	
3067	7300	EXTON, CLA CLL	/ SO ADD THEM
3070	1354	TAD EXTCNT	
3071	1167	TAD EXTRA	
3072	7700	SMA CLA	
3073	5350	JMP EXFINI	
3074	1355	TAD XOSEXT	
3075	5776	JMP I SETS	
3076	2356	ISZ XOSEXT	
3077	1755	TAD I SOXEXT	
3100	7040	CMA	
3101	3113	DCA DV1	
3102	1756	TAD I SOXEXT	
3103	7450	SNA	
3104	5310	JMP .+4	
3105	7041	CIA	
3106	3114	DCA DV2	
3107	5312	JMP .+3	
3110	2113	ISZ DV1	
3111	3114	DCA DV2	
3112	1114	TAD DV2	
3113	1110	TAD QUOT2	
3114	3114	DCA DV2	
3115	7004	RAL	
3116	1113	TAD DV1	
3117	1107	TAD QUOT1	
3120	3113	DCA DV1	
3121	7100	CLL	
3122	1117	TAD DVDPT	
3123	3327	DCA EXTML+1	
3124	1117	TAD DVDPT	
3125	3330	DCA EXTML+2	
3126	4521	EXTML, JMS I DMULPT	/ SQUARE DOUBLE
3127	0000	0	/ PRECISION
3130	0000	0	
3131	0000	SZA CLA	
3132	4553	JMS I CNTRO	
3133	1532	TAD I DV2PT	
3134	7640	SZA CLA	
3135	4553	JMS I CNTRO	
3136	1560	TAD I RICNT	

3137	3757		DCA I EXTSOX	/ AND DEPOSIT THEM
3140	2357		ISZ EXTSOX	
3141	1561		TAD I R2CNT	
3142	3757		DCA I EXTSOX	
3143	2357		ISZ EXTSOX	
3144	2354		ISZ EXTCNT	
3145	2355		ISZ XOSEXT	
3146	2355		ISZ XOSEXT	
3147	5267		JMP EXTON	
3150	1167	EXFINI,	TAD EXTRA	
3151	3106		DCA CNTR	
3152	5651		JMP I NEGIT	
3153	7767	MD9,	-9	
3154	0000	EXTCNT,	0	
3155	0000	XOSEXT,	0	
3156	0000	SOXEXT,	0	
3157	0000	EXTSOX,	0	
3160	0000	TEST,	0000	/ VALID VALUES ?
3161	1246		TAD M260	
3162	7550		SPA SNA	
3163	5371		JMP QUES	/ NO
3164	1374		TAD MN7	
3165	7540		SMA SZA	
3166	5371		JMP QUES	/ NO
3167	1375		TAD PL7	
3170	5760		JMP I TEST	/ YES
3171	7300	QUES,	CLA CLL	
3172	4446		JMS I CRLF	
3173	5217		JMP ONGO+1	
3174	7771	MN7,	-7	
3175	0007	PL7,	7	
3176	2355	SETS,	2355	

3200	0000	*3200	0000	
3201	7300	DELEIT,	CLA CLL	/ DELETE A RATIO
3202	1032		TAD XORSOR	
3203	1327		TAD MM331	
3204	7650		SNA CLA	
3205	4572		JMS I STAR70	
3206	1032		TAD XORSOR	
3207	1325		TAD MM304	
3210	7640		SZA CLA	
3211	5600		JMP I DELEIT	
3212	5731	OKAY,	JMP I CHECK	/ VALIDITY CHECK
3213	7041		CIA	
3214	3333		DCA NCNTR	
3215	3334		DCA MUMCNT	
3216	4453		JMS I TAB	
3217	4453		JMS I TAB	
3220	7300	DELAGN,	CLA CLL	/ WHICH RATIO TO
3221	1336		TAD DELCH	/ DELETE ?
3222	4433		JMS I WRITE	
3223	7771		-7	
3224	6032		KCC	
3225	4450		JMS I LISN	
3226	1032		TAD XORSOR	
3227	3101		DCA IEMTS	
3230	1106		TAD CNTR	
3231	1346		TAD CNTCTR	
3232	1032		TAD XORSOR	
3233	7540		SNA SZA	/ WAS IT CORRECT ?
3234	5263		JMP DELWR	
3235	1333		TAD NCNTR	
3236	7550		SPA SNA	
3237	5263		JMP DELWR	
3240	5732	FINE,	JMP I CHCIT	/ YES , SO DELETE IT
3241	1335		TAD DELLCN	
3242	1335		TAD DELLCN	
3243	1103		TAD LOCTON	
3244	1324		TAD MN2	
3245	3335		DCA DELLCN	
3246	3735		DCA I DELLCN	
3247	1335		TAD DELLCN	
3250	1330		TAD P400	
3251	3347		DCA LCNDEL	
3252	3747		DCA I LCNDEL	
3253	2335		DCA DELLCN	
3254	2347		ISZ LCNDEL	
3255	3735		DCA I DELLCN	

3256	3747		DCA I LCNDEL	
3257	2334		ISZ MUMCNT	
3260	4446		JMS I CRLF	
3261	4576		JMS I TAB50	
3262	5220		JMP DELAGN	
3263	7300	DELWR,	CLA CLL	/ NO, SO ASK AGAIN
3264	4446		JMS I CRLF	
3265	1032		TAD XORSOR	
3266	1326		TAD MM316	
3267	7650		SNA CLA	
3270	5274		JMP .+4	
3271	4451		JMS I BELL	
3272	4576		JMS I TAB50	
3273	5220		JMP DELAGN	
3274	4446	DELOUT,	JMS I CRLF	/ SET UP COUNTERS
3275	4446		JMS I CRLF	/ FOR EVALUATION
3276	1077		TAD TEMTQ	
3277	1334		TAD MUMCNT	
3300	3077		DCA TEMTQ	
3301	2350		ISZ MUMOK	
3302	1103		TAD LOCTON	
3303	3756		DCA I NXOSLL	
3304	3757		DCA I NSTRGE	
3305	3760		DCA I NSTAGE	
3306	1334		TAD MUMCNT	
3307	7041		CIA	
3310	1752		TAD I CONT1	
3311	3752		DCA I CONT1	
3312	1334		TAD MUMCNT	
3313	7041		CIA	
3314	7000		NOP	
3315	3753		DCA I CONT2	
3316	1354		TAD CONT3	
3317	3755		DCA I CONT4	
3320	2361		ISZ NUMCTR	
3321	1076		TAD TEMTP	
3322	3106		DCA CNTR	
3323	5362		JMP NEG	
3324	7776	MN2,	-2	
3325	7474	MM304,	-304	
3326	7462	MM316,	-316	
3327	7447	MM331,	-331	
3330	0400	P400,	400	
3331	3420	CHECK,	3420	
3332	3436	CHCIT,	3436	
3333	0000	NCNTR,	0	
3334	0000	MUMCNT,	0	
3335	0000	DELLCN,	0	
3336	3336	DELCH,	.	/ -7 (-7 DEC)
3337	0304		304	/ DELETE

3240	0305		305	
3241	0314		314	
3242	0305		305	
3243	0324		324	
3244	0305		305	
3245	0240		240	
3246	7520	CNTCTR,	-260	
3247	0000	LCNDEL,	0000	
3250	0000	MUMOK,	0000	
3251	4047	CONT,	4047	
3252	4142	CONT1,	4142	
3253	0045	CONT2,	0045	
3254	5776	CONT3,	5776	
3255	4130	CONT4,	4130	
3256	4145	NXOSLL,	4145	
3257	4146	NSTRGE,	4146	
3260	4147	NSTAGE,	4147	
3261	0000	NUMCTR,	0000	
3262	1372	NEG,	TAD MX3	/ NEGATE
3263	3767		DCA I NEXTRA	
3264	1370		TAD NLCT	
3265	3771		DCA I NGLCT	
3266	5751		JMP I CONT	
3267	0167	NEXTRA,	0167	
3270	2405	NLCT,	2405	
3271	3051	NGLCT,	3051	
3272	0000	MX3,	0000	
3273	3520	SET,	3520	

3400	0000	*3400	0000	/ DCA 0 IN RATIO TO BE
3401	1614	CRTMUM,	TAD I NMUMOK	/ DELETED
3402	7650		SNA CLA	
3403	5600		JMP I CRTMUM	
3404	3614		DCA I NMUMOK	
3405	1215		TAD PL6	
3406	3616		DCA I NK6	
3407	1314		TAD PL3	
3410	3617		DCA I NK3	
3411	1315		TAD K1106	
3412	3716		DCA I N4130	
3413	5600		JMP I CRTMUM	
3414	3350	NMUMOK,	3350	
3415	0006	PL6,	6	
3416	4142	NK6,	4142	
3417	0045	NK3,	0045	
3420	7300	CHECK,	CLA CLL	/ SET COUNTERS
3421	1106		TAD CNTR	
3422	1235		TAD DP6	
3423	7650		SNA CLA	
3424	5231		JMP .+5	
3425	1106		TAD CNTR	
3426	3076		DCA TEMTP	
3427	1044		TAD M3	
3430	5234		JMP .+4	
3431	1106		TAD CNTR	
3432	3076		DCA TEMTP	
3433	1106		TAD CNTR	
3434	5665		JMP I OKAY+1	
3435	0006	DP6,	6	
3436	3666	CHCIT,	DCA I DELCN	/ DEPOSIT ZEROS
3437	1106		TAD CNTR	
3440	1235		TAD DP6	
3441	7650		SNA CLA	
3442	5244		JMP .+2	
3443	5256		JMP .+11	
3444	1667		TAD I NCNTRN	
3445	3100		DCA TEMTR	
3446	5670		JMP I FINE+1	
3447	1100		TAD TEMTR	
3450	1666		TAD I DELCN	
3451	3666		DCA I DELCN	
3452	1100		TAD TEMTR	
3453	1045		TAD K3	
3454	3100		DCA TEMTR	
3455	5670		JMP I FINE+1	

3456	1076		TAD TEMTR
3457	7000		NOP
3460	1100		TAD TEMTR
3461	7650		SNA CLA
3462	5264		JMP .+2
3463	5247		JMP .-12
3464	5272		JMP DELOTE
3465	3213	OKAY+1,	3213
3466	3335	DELCN,	3335
3467	3333	NCTNRN,	3333
3470	3241	FINE+1,	3241
3471	1442	DELOT,	1442
3472	4446	DELOTE,	JMS I CRLF
3473	4446		JMS I CRLF
3474	5671		JMP I DELOT

3500	0000	TABA50,	0000	/ TAB 50 SPACES
3501	7300		CLA CLL	
3502	1311		TAD M50	
3503	3312		DCA LCN50	
3504	1313		TAD KS240	
3505	4447		JMS I TYPE	
3506	2312		ISZ LCN50	
3507	5304		JMP .-3	
3510	5700		JMP I TABA50	
3511	7730	M50,	-50	
3512	0000	LCN50,	0	
3513	0240	KS240,	240	
3514	0003	PL3,	3	
3515	1106	K1106,	1106	
3516	4130	N4130,	4130	

3517	0000			
3520	7300		CLA CLL	/ SORT LOCATIONS
3521	1747		TAD I CNTC	
3522	1346		TAD PL3	
3523	3747		DCA I CNTC	
3524	1345		TAD X	
3525	7640		SZA CLA	
3526	5331		JMP .-3	
3527	5366		JMP MUMCT+1	
3530	5744		JMP I CONTX	
3531	5372		JMP MUMCT+5	
3532	7300		CLA CLL	
3533	3345		DCA X	
3534	5743		JMP I PAROUT	

3543	4000	PAROUT,	PARM	/ RESET TO START
3544	4136	CONTX,	4136	/ OVER COMPLETE
3545	0000	X,	0000	/ PROGRAM

3546	0003	PL3,	3	
3547	3346	CNTC,	3346	
3550	0000	CRNUMT,	0000	/ SET START COUNTERS
3551	7300		CLA CLL	
3552	1764		TAD I NUMCT	
3553	7650		SNA CLA	
3554	5750		JMP I CRNUMT	
3555	1765		TAD I MUMCT	
3556	7041		CIA	
3557	1105		TAD NUMT	
3560	3105		DCA NUMT	
3561	3764		DCA I NUMCT	
3562	7300		CLA CLL	
3563	5750		JMP I CRNUMT	
3564	3361	NUMCT,	3361	
3565	3334	MUMCT,	3334	
3566	1747		TAD I CNTC	
3567	1346		TAD PL3	
3570	3747		DCA I CNTC	
3571	2345		ISZ X	
3572	1106		TAD CNTR	
3573	1346		TAD PL3	
3574	3776		DCA I MXM3	
3575	5330		JMP PAROUT	.-13
3576	3372	MXM3,	3372	

		*4000		
4000	7300	PARM,	CLA CLL	/ START OF MAINLINE
4001	3106		DCA CNTR	/ SET COUNTERS
4002	3167		DCA EXTRA	
4003	3164		DCA NEWXOS	
4004	1104		TAD LOCTEN	
4005	3566		DCA I BLAH	
4006	4353		JMS COMMET	/ START OUTPUT FORMAT
4007	4446		JMS I CRLF	
4010	4552		JMS I GOON	
4011	4541		JMS I DPCHAG	
4012	1103		TAD LOCTON	
4013	3345		DCA LOCTON	
4014	3346		DCA STORGE	
4015	3347		DCA STOAGE	
4016	3351		DCA MEANCT	
4017	5226		JMP MEANS	
4020	2351	MBES,	ISZ MEANCT	/ ASK HOW MANY TO DO
4021	1037		TAD CHAR04	
4022	4433		JMS I WRITE	
4023	7753		-25	
4024	4446		JMS I CRLF	
4025	5226		JMP MEANS	
4026	1351	MEANS,	TAD MEANCT	/ AND COLLECT DATA
4027	7450		SNA	
4030	5220		JMP MBES	
4031	1343		TAD M9	
4032	7700		SMA CLA	
4033	4240		JMS NMEANS	
4034	2351		ISZ MEANCT	
4035	4533		JMS I SETIS	/ AND SORT IT
4036	4535		JMS I DEPOST	/ AND DEPOSIT IT
4037	5226		JMP MEANS	
4040	0000	NMEANS,	0000	/ DO MEAN RATIO
4041	1106		TAD CNTR	
4042	7640		SZA CLA	
4043	5246		JMP .+3	
4044	5372		JMP CLEAR	
4045	5247		JMP MEANON	
4046	4546		JMS I AGAIN	
4047	7300	MEANON,	CLA CLL	
4050	3350		DCA STOCNT	
4051	1350	SUMTOT,	TAD STOCNT	/ FROM STORAGE
4052	1106		TAD CNTR	
4053	7700		SMA CLA	
4054	5273		JMP SUMMON	

4055	1345		TAD XOSLL	
4056	3352		DCA FIXS	
4057	2352		ISZ FIXS	
4060	1346		TAD STORGE	
4061	1752		TAD I FIXS	
4062	3346		DCA STORGE	
4063	7004		RAL	
4064	1347		TAD STOAGE	
4065	1745		TAD I XOSLL	
4066	3347		DCA STOAGE	
4067	2345		ISZ XOSLL	
4070	2345		ISZ XOSLL	
4071	2350		ISZ STOCNT	/ ALL DONE ?
4072	5251		JMP SUMTOT	/ NO
4073	1346	SUMMON,	TAD STORGE	/ YES , SO DO
4074	3116		DCA DV4	/ THE MEAN OF THEM
4075	1347		TAD STOAGE	
4076	3115		DCA DV3	
4077	3113		DCA DV1	
4100	3114		DCA DV2	
4101	3111		DCA DVSOR1	
4102	1106		TAD CNTR	
4103	1342		TAD K6	
4104	7640		SZA CLA	/ 9 OR 6 VALUES ?
4105	5312		JMP .+5	
4106	1342		TAD K6	/ 6 TO AVERAGE
4107	3344		DCA MUM	
4110	3106		DCA CNTR	
4111	5315		JMP READI	
4112	1344		TAD MUM	
4113	1045		TAD K3	/ 9 TO AVERAGE
4114	3344		DCA MUM	
4115	1344	READI,	TAD MUM	/ SO PRINT THEM
4116	3112		DCA DVSOR2	/ AND DO RATIO
4117	4556		JMS I DVONE	/ MEAN
4120	1040		CHAR05	
4121	4433		JMS I WRITE	
4122	7765		-13	
4123	1127		TAD QUOTPT	
4124	3326		DCA ADDRS3	
4125	4465		JMS I DECPRT	/ PRINT MEAN
4126	0000	ADDRS3,	0000	
4127	4446		JMS I CRLF	
4130	1106		TAD CNTR	
4131	7640		SZA CLA	
4132	5777		JMP I FIX	
4133	4550		JMS I STDDEV	/ DO STANDARD
4134	4543		JMS I INCASE	/ DEVIATION NOW
4135	5340		JMP .+3	
4136	4542		JMS I STDTW	/ REPEAT FOR THREE
4137	4543		JMS I INCASE	/ MORE SETS

4140	4545		JMS I HAPPY	/ FINISHED THIS
4141	4572		JMS I STAR70	/ SAMPLE ?
4142	0006	K6,	6	
4143	7767	M9,	-11	
4144	0000	MUM,	0	
4145	0000	XOSLL,	0	
4146	0000	STORGE,	0	
4147	0000	STOAGE,	0	
4150	0000	STOCNT,	0	
4151	0000	MEANCT,	0	
4152	0000	FIXS,	0	
4153	0000	COMMET,	0000	/ ONE LINE FOR
4154	6046		TLS	/ COMMENTS
4155	4562		JMS I XOSMB	
4156	4446		JMS I CRLF	
4157	7300		CLA CLL	
4160	4450		JMS I LISN	
4161	1032		TAD XORSOR	
4162	1371		TAD MN215	
4163	7640		SZA CLA	/ ENDS WITH CRLF
4164	5357		JMP .-7	
4165	4446		JMS I CRLF	
4166	4446		JMS I CRLF	
4167	3102		DCA TEMTT	
4170	5753		JMP I COMMET	
4171	7563	MN215,	-215	
4172	7300	CLEAR,	CLA CLL	
4173	3573		DCA I CNTXOS	
4174	4534		JMS I RATIOS	
4175	5247		JMP MEANON	
4176	3052	NEGIT+1,	3052	
4177	3520	FIX,	3520	

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